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# Kinetic mechanism and specificity of phosphorylase kinase

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Kinetic mechanism and specificity of  
phosphorylase kinase

by

Louisa Braal Tabatabai

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## ABBREVIATIONS

AMPPCP	Adenylyl ( $\beta,\gamma$ -methylene)-diphosphate
ATP	Adenosine triphosphate
cyclic AMP	3' - 5' - Cyclic adenosine monophosphate
DTE	Dithioethrythritol
EDTA	Ethylenediaminetetraacetate
EGTA	Ethyleneglycol-bis( $\beta$ -aminoethyl ether)-N,N'-tetraacetic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid
ITP	Inosine triphosphate
MOPS	Morpholinopropane sulfonic acid
PIPES	Piperazine-N,N'-bis (2-ethane sulfonic acid)
TCA	Trichloroacetic acid
TES	(N-tris [Hydroxymethyl] methyl-2-aminoethane sulfonic acid
SDS	Sodium dodecyl sulfate

## INTRODUCTION

Muscle phosphorylase b kinase (ATP:phosphorylase phosphotransferase EC 2.7.1.38) is a specific protein kinase which catalyzes the conversion of phosphorylase b to phosphorylase a in the presence of divalent metal ions and ATP (Fischer and Krebs, 1955; Krebs and Fischer, 1956; Kent et al., 1958). In this reaction, the terminal phosphate group of ATP is transferred to a specific serine residue located in each of the two identical N-terminal segments of the dimeric phosphorylase molecule (Fischer et al., 1959; Nolan et al., 1964). This covalent modification of phosphorylase activates the enzyme.

Phosphorylase b kinase itself also exists in two interconvertible forms (Krebs et al., 1959; Riley et al., 1968); these forms are referred to as nonactivated phosphorylase b kinase (dephospho-enzyme) and activated phosphorylase b kinase (phospho-enzyme) by Krebs et al., (1964). Activation of phosphorylase b kinase in vivo occurs after hormonal stimulation or during muscle contraction. Hormone mediated activation of kinase is catalyzed by cyclic AMP-dependent protein kinase (DeLange et al., 1968; Possner et al., 1965; Walsh et al., 1968). This reaction which requires the presence of cyclic AMP, ATP and  $Mg^{2+}$  ions is the step through which epinephrine influences phosphorylase a formation and glycogenolysis

(Possner et al., 1965). Glycogenolysis is also observed after the onset of muscle contraction. Electrical stimulation of muscle causes the release of  $\text{Ca}^{2+}$  ions from the sarcoplasmic reticulum, which then initiates muscle contraction (Ozawa et al., 1967). During this process there is a rapid conversion of phosphorylase b to a concomitant with glycogenolysis, but without an increase in cyclic AMP levels (Danforth et al., 1962; Possner et al., 1965). Brostrom et al., (1971) demonstrated that phosphorylase kinase, which has an absolute requirement for  $\text{Ca}^{2+}$  ions, can convert phosphorylase to the active form in the absence of cyclic AMP. However, phosphorylase conversion was inhibited reversibly by the addition of the physiological calcium chelator, sarcoplasmic reticulum. Several investigators hypothesized in the light of these experiments that phosphorylase kinase plays a key role in linking muscle contraction to glycogenolysis (Brostrom et al., (1971); Ozawa et al., 1967).

#### Properties of Phosphorylase b Kinase

In the following discussion phosphorylase kinase from muscle tissue will be discussed exclusively, unless otherwise indicated. Aspects of kinase such as physicochemical properties, regulation of kinase activity by pH,  $\text{Ca}^{2+}$ , glycogen and phosphorylation state will be discussed first. And finally, the kinetic properties of both forms of kinase will be

considered.

### Physicochemical properties of kinase

Phosphorylase kinase in rabbit skeletal muscle represents approximately 0.8% of the soluble protein (Cohen, 1973; DeLange et al., 1968). Assuming it is uniformly distributed in the muscle cell, its concentration would be close to 1 mg/ml. It has been reported to be present at a concentration of almost 15% by weight of its substrate phosphorylase (Cohen, 1973). The physiological significance of this is not understood.

Phosphorylase kinase has been isolated from rabbit muscle in a homogeneous form having a sedimentation coefficient  $S_{20,w}^0$  of 23-26 (Cohen, 1973; Hayakawa et al., 1973). The molecular weight has been estimated to be  $1.33 \times 10^6$  by these investigators, and the absorption coefficient,  $A_{280}^{1\%}$ , has a value of 12.4 and 11.8; and the ratio at 280 to 260 nm of the enzyme is 1.90 and 1.75 as determined by Cohen (1973) and Hayakawa et al., (1973), respectively.

Phosphorylase kinase is an oligomeric enzyme consisting of 3 types of polypeptides, A, B and C of molecular weights 118,000, 108,000 and 41,000, respectively (Hayakawa et al., 1973) as estimated from disc gel electrophoresis in the presence of SDS. From the relative ratios of the stained protein bands and the molecular weight of the subunits, the

composition of subunits was calculated to be  $A_4 B_4 C_8$ . Cohen (1973), relying on densitometric analysis of gels, gel filtration on Sephadex G-200 in the presence of SDS, and by carboxymethylation of sulfhydryl groups with iodo [ $^{14}C$ ] acetate, found on the other hand, that the enzyme exists as a dodecamer with a composition of 4(ABC) with a minimal molecular weight of 318,000. The subunits A, B and C have molecular weights of 145,000, 128,000 and 45,000. In addition to the three subunits described, there is always present a fourth protein band on SDS gels called A', of molecular weight 140,000 (Cohen, 1973). Originally, it was thought that A' was a fragment produced from the A subunit by limited proteolysis (Cohen, 1973), but more recently evidence has been produced that more likely it represents a subunit of an isozyme of phosphorylase kinase of red muscles (Jennissen and Heilmeyer, 1974).

It is still open to speculation as to exactly which roles the subunits play in the expression of kinase activity. Phosphorylase kinase obtained after limited attack by trypsin was still found to be catalytically active (Krebs et al., 1964; Huston and Krebs, 1968). Subunit structure of phosphorylase kinase exposed to limited trypsin attack consisted of degraded A and B subunits, and an apparently intact C subunit as first shown by Graves et al., (1973) and Hayakawa et al., 1973 and later by Cohen, 1973. This

partially degraded phosphorylase kinase showed increased activity as evidenced by an increase in the ratio of activities measured at pH 6.8 to 8.2 (Hayakawa et al., 1973). Graves et al., (1973) showed that trypsin modified phosphorylase kinase could be dissociated in the presence of ATP to give catalytically active species. Identification of fractions isolated by sucrose gradient centrifugation showed the presence of an active 6S fraction by gel electrophoresis containing only the C subunit and degraded material. In view of these results and those of Hayakawa et al., (1973) it was suggested that the catalytic subunit may be located on the C subunit and that the A subunit or a derived form is not necessary for catalysis of phosphorylase kinase. However, since partially degraded B subunit components were present in the dissociated form of phosphorylase kinase containing the intact C subunit, it could not be stated with certainty which components were necessary for catalytic activity (Graves et al., 1973).

#### Regulation of phosphorylase kinase activity

Activity of phosphorylase kinase is influenced by various factors. Among these factors are calcium ion concentration, pH, glycogen, and the activation state of kinase (i.e. degree of phosphorylation of kinase subunits). Each of these conditions will be considered in detail below.

### Regulation by $\text{Ca}^{2+}$ ions

Conversion of phosphorylase b to a after passing crude muscle extracts through unwashed filter paper was first observed by Fischer and Krebs (1955). They established that this effect was due to the extraction of metals from the paper. The reaction of phosphorylase b to a itself, however, was found to require  $\text{Mg}^{2+}$ , or  $\text{Mn}^{2+}$  and ATP (Krebs and Fischer, 1956). It wasn't until 1959 that Krebs et al. discovered a role for calcium in the activation of phosphorylase kinase. These investigators showed that incubation of kinase with  $\text{Ca}^{2+}$  ions changes the kinase from a pH 7 inactive form to a form which has now considerable activity at pH 7.0. No reversal of the  $\text{Ca}^{2+}$  activation of phosphorylase kinase could be demonstrated after extensive dialysis against EDTA or after addition of a 2-fold molar excess of EDTA over  $\text{Ca}^{2+}$ . Meyer et al., (1964) discovered a protein factor responsible for the observed  $\text{Ca}^{2+}$  activation of phosphorylase kinase. However, phosphorylase kinase free of this factor had a poor response to  $\text{Ca}^{2+}$  activation, and recombinations of column fractions restored this response. Subsequently, a protein factor was isolated and found to be involved in the  $\text{Ca}^{2+}$  mediated activation of kinase. Because of the irreversibility of the activation reaction, and because the protein factor was shown to be a proteolytic enzyme (Huston and Krebs, 1968), it was concluded by these investigators that



this mechanism of activation of phosphorylase kinase was of no physiological significance. However, Meyer et al., (1964) made the observation that the phosphorylase kinase reaction itself was subject to inhibition by chelating agents. They observed that EGTA, a specific  $\text{Ca}^{2+}$  chelating agent, was a more effective inhibitor of kinase than EDTA. Furthermore, inhibition by EGTA was found to be reversed by excess  $\text{Ca}^{2+}$  ions. From these observations they first postulated that  $\text{Ca}^{2+}$  ions might provide the link coupling muscle contraction to glycogenolysis. Ozawa et al., (1967) investigated the  $\text{Ca}^{2+}$  requirement for stimulation of phosphorylase activity, as it was known to them that less than  $3 \times 10^{-6}$  M  $\text{Ca}^{2+}$  was required for maximal muscle tension development. They concluded from EGTA titration curves that half maximal activity of phosphorylase kinase occurred at  $10^{-7}$  M free  $\text{Ca}^{2+}$ . Brostrom et al., (1971) using highly purified rabbit muscle phosphorylase kinase and utilizing calcium-free reagents, reported apparent  $K_m$  values of  $\text{Ca}^{2+}$  for activated kinase ( $2 \times 10^{-7}$  M at pH 8.2 and  $5 \times 10^{-7}$  M at pH 6.8) and for nonactivated kinase ( $3 \times 10^{-6}$  M at pH 8.2 and indeterminate at pH 6.8) which are in the range of free  $\text{Ca}^{2+}$  levels in muscle. Intracellular free  $\text{Ca}^{2+}$  levels of resting muscle were estimated to be  $10^{-7}$  M and  $1-3 \times 10^{-5}$  M to  $3 \times 10^{-6}$  M for muscle under maximal tension (Ebashi et al., 1969; Ozawa et al., 1967). Brostrom et al., (1971) also

showed that the naturally occurring calcium chelator of muscle, sarcoplasmic reticulum, could instantaneously arrest the phosphorylase b to a conversion catalyzed by phosphorylase kinase in in vitro experiments. Addition of calcium reversed the inhibitory effect of sarcoplasmic reticulum. Complete dependence of phosphorylase kinase activity on calcium ions was also demonstrated in experiments using intact protein-glycogen particles (Heilmeyer et al., (1970). The protein-glycogen particle has been isolated as an entity from rabbit skeletal muscle, containing phosphorylase b, phosphorylase kinase, and phosphorylase phosphatase, among other proteins (Heilmeyer et al., 1970). The half-maximum activation of kinase in this system required  $2 \times 10^{-6}$  M free calcium in contrast to  $10^{-7}$  M calcium for purified kinase solutions. The calcium concentration needed to cause conversion of phosphorylase b to a in the glycogen particle was found to be the same as that needed (as mentioned earlier) to trigger muscle contraction (Heilmeyer et al., 1970). These results, therefore, suggest that calcium ions provide the link between the onset of muscle contraction and initiation of glycogenolysis by phosphorylase kinase.

#### Regulation of activity by glycogen

Regulation of phosphorylase kinase activity by glycogen was studied in a glycogen particle derived from muscle.

Heilmeyer et al., (1970) reported that the protein-glycogen complex contains the enzymes of glycogen metabolism as well as those of the glycolytic pathway. One aspect of the regulation of kinase activity in the glycogen particle, as mentioned earlier, was that the  $K_m$  of  $Ca^{2+}$  for kinase was higher than the  $K_m$  of  $Ca^{2+}$  for purified kinase. Destruction of the particle with amylase increased the affinity of kinase for  $Ca^{2+}$  to that observed with purified enzyme. Earlier work reported by Krebs et al., (1964) not only considered the effects of glycogen on phosphorylase kinase activity, but also the effect glycogen would have on the ability of phosphorylase to serve as substrate for kinase. They believed that the observed decrease in the  $K_m$  of phosphorylase b in the presence of glycogen could be interpreted to mean that the glycogen-phosphorylase b complex had a greater affinity for the kinase. However, it could not be ruled out that significant enzyme activation occurred as well, since glycogen was also shown to have a stimulatory effect on kinase activation by MgATP. Although the glycogen particle is a very complex system, it appears to be an interesting model for studying mechanism of regulation of phosphorylase kinase.

#### Dependence of activity on pH

As discussed in a previous section, phosphorylase b kinase as extracted from resting muscle is present almost

totally in the inactive form. Activity of kinase at pH 7 or below is very low because of its low affinity for phosphorylase at this pH value (Krebs et al., 1964). From studies of the pH dependence of the kinase reaction, Krebs et al., (1959,1964) have shown that optimal activity of non-activated kinase is at approximately pH 8.5 in a buffer system containing  $\beta$ -glycerophosphate and Tris. In an earlier attempt to measure the pH dependence of the kinase reaction, Krebs and Fischer (1956) reported that activity of kinase depended on which buffer was used. Phosphorylase kinase had higher activities in  $\beta$ -glycerophosphate and Tris, than in Tris alone. This effect by Tris was thought to be due to this buffer binding divalent metal ions which are essential for kinase activity.

Phosphorylase kinase activities are often expressed as a ratio of activities at pH 6.8 to 8.2. This ratio is usually in the range of 0.01 to 0.05 for nonactivated kinase and up to 1.0 for various forms of activated kinase (Cohen, 1973; Krebs et al., 1964). The increase in ratio is largely a result of increased activity of kinase at pH 6.8. For example, protein kinase activated phosphorylase kinase has a pH 6.8:8.2 ratio of 0.36; the 6.8 activity increased 10 to 15-fold and the 8.2 activity 1.5 to 2-fold (Cohen, 1973).

### Effect of phosphorylation on kinase activity

Phosphorylase kinase can be activated through covalent modification by phosphorylation of specific seryl residues on subunits A and B but not C. Two mechanisms for covalent modification of phosphorylase kinase have been reported in the literature. The first is an in vivo mechanism of activation of phosphorylase kinase which occurs after a hormonal mediated stimulus, and which results in the well-known cascade of reactions leading to glycogenolysis in muscle as well as in liver (for an up-to-date review see Krebs and Preiss, 1975). The second mechanism, which is autocatalytic, has been demonstrated to occur in vitro in the presence of  $\text{Ca}^{2+}$  ions and MgATP (DeLange et al., 1968; Walsh et al., 1971; Hayakawa et al., 1973; Carlson and Graves, 1976; Wang et al., 1976). Whether this mechanism occurs in vivo is not yet known. Both mechanisms of activation of phosphorylase kinase will be considered separately.

The enzyme responsible for activation and phosphorylation of phosphorylase kinase in the hormone stimulated process is cyclic AMP-dependent protein kinase (Walsh et al., 1968). This reaction takes place in the presence of  $\mu\text{M}$  amounts of cyclic AMP at low ATP levels (0.1-0.4 mM), pH 6.8, and is not inhibited by EGTA (Cohen, 1973; Hayakawa et al., 1973). Extensive studies on subunit phosphorylation in the presence of cyclic AMP-dependent protein kinase were presented by these

workers. They reported that most of the change in enzyme activity occurs during phosphorylation of the B subunit. Phosphorylation of the A subunit showed a lag phase until the B subunit phosphorylation was 50% complete. Enzyme activity corresponded closely to the B subunit phosphorylation. The rate of activation leveled off when approximately 1 mole of phosphate was incorporated in each of the subunits A and B (Cohen, 1973); no phosphate was incorporated in the C subunit. The fact that only one mole of phosphate is incorporated per mole of A and B subunits, indicates that this reaction is a specific one. Two out of a possible 179 serine residues are covalently modified by the cyclic AMP-dependent protein kinase reaction (Cohen, 1973). Tryptic phosphopeptides isolated from the A and B subunits have the following sequences, respectively: arg-leu-ser(P)-ile-ser-thr-glu-ser-glx-pro-asx-gly and arg-ser-gly-ser(P)<sup>val</sup><sub>ile</sub>-try-glu-pro-leu-lys (Cohen, personal communication; Yeaman and Cohen, 1975). The ambiguity present in the latter peptide is due to the presence of two alleles for the B subunit in the muscle from the rabbit population used (Cohen et al., 1975). These same phosphopeptides isolated from in vitro phosphorylation experiments corresponded exactly to those isolated from phosphorylase kinase activated in vivo after adrenalin injection of rabbits (Yeaman and Cohen, 1975), thus providing final proof for the role of phosphorylase kinase in the hormonal control of

skeletal muscle glycogenolysis.

Phosphorylation and activation of phosphorylase kinase can also occur in the absence of cyclic AMP-dependent protein kinase. This mechanism of activation of kinase, which occurs in the presence of calcium, magnesium and about 10-fold higher concentrations of ATP than the protein kinase catalyzed reaction, is inhibited by EGTA and is reportedly catalyzed by phosphorylase kinase itself (Krebs et al., 1964; DeLange et al., 1968; Carlson and Graves, 1976; Hayakawa et al., 1973. Walsh et al., 1971 and Wang et al., 1976). The stoichiometry of phosphate incorporation into phosphorylase kinase subunits through autophosphorylation was much greater than that reported to occur by protein kinase catalyzed phosphorylation. Up to 5 moles of phosphate in A and 2 moles of phosphate in the B subunit were incorporated in the autophosphorylation reaction in the presence of Mg and ATP (10 mM and 3.3 mM, respectively) and an alternative substrate, a tetradecapeptide derived from phosphorylase b (Carlson and Graves, 1976). It can be calculated that a total of 28 moles of phosphate were incorporated per mole of enzyme. In a separate experiment described by these authors, the pH 6.8 activity of non-activated phosphorylase kinase was measured during autophosphorylation. A 13-fold increase in pH 6.8 activity was observed when autophosphorylation was performed in the presence of peptide substrate, and a 4-fold increase in pH 6.8 activity

(1976) found that autoactivation in the presence of high levels of  $Mg^{2+}$  and ATP (10 mM and 3 mM, respectively) resulted in the incorporation of 7-9 moles of phosphate per ABC unit of enzyme, or a total of up to 36 moles of phosphate per  $A_4B_4C_4$  unit of enzyme. These authors observed a 2-fold increase in the pH 6.8 activity of the enzyme. These seemingly confusing results could be explained perhaps by the kind of buffer used in the autophosphorylation and autoactivation experiments. The conventionally used buffer  $\beta$ -glycerophosphate was found to inhibit autophosphorylation and autoactivation, while HEPES (Carlson and Graves, 1976) and Mes and Tes buffers (Wang et al., 1976) activated these events. Another interesting aspect of the autoactivation reaction is that fully autoactivated phosphorylase kinase could not be further activated by protein kinase, although the former could be further phosphorylated (Wang et al., 1976). These authors also reported that the reverse sequence, i.e., protein kinase-catalyzed activation followed by autocatalytic activation, did result in increased activity as well as phosphorylation. Whether or not the mechanism of autoactivation is important physiologically is open to speculation. Phosphorylase kinase and phosphorylase are closely associated in the glycogen particle in vivo. Presumably, this association could cause a large stimulation of activity and autophosphorylation once  $Ca^{2+}$  became available (Carlson and Graves, 1976).



Furthermore, Danforth et al., (1962) found that there were two in vivo mechanisms for activating phosphorylase kinase, one triggered by hormonal stimulus (epinephrine) and another through muscle contraction (neural excitation), which causes the release of  $\text{Ca}^{2+}$ . Wang et al., (1976) from additional experiments found, that phosphate and organic phosphates (phosphorylated compounds in the glycolytic pathway) suppressed the autocatalytic event. On the basis of these results they concluded that if autoactivation does play a role, conditions and/or substances which would shorten the overall slow response of the enzyme or which would counteract the phosphate effect, would probably have to exist. No evidence is available so far on the sites phosphorylated by the autocatalytic mechanism. Wang et al., (1976) suggested on the basis of their results, that the sites of phosphorylase kinase phosphorylation and protein kinase-catalyzed phosphorylation are nonoverlapping. This is based on the observation by these investigators that the extent of phosphorylase kinase phosphorylation by these two reactions are approximately additive.

#### Kinetic properties of phosphorylase kinase

In this section the kinetic parameters of the kinase reaction will be discussed as far as they are known. A matter which would complicate interpretation of results for

nonactivated kinase is the fact that the rate of product formation is not linear with time. This is especially evident at pH values below pH 7.7 (Krebs et al., 1959). When nonlinear progress curves are obtained, then obviously, a constant slope cannot be calculated, and therefore no estimate can be made of initial velocity, which represents amount of product formed per unit time. Therefore, plots of the reciprocal of initial velocity versus the reciprocal of substrate concentration, from which the  $K_m$  is determined, cannot be constructed. Thus kinetic parameters can only be measured when the following conditions are satisfied. First, the rate of product formation must be linear and secondly, the initial velocity must vary linearly with enzyme concentration. The latter condition is implicit in the Michaelis-Menten equation. Nevertheless, some of the kinetic parameters of the phosphorylase kinase reaction have been reported. Krebs and coworkers (1964) have studied the dependence of the  $K_m$  of phosphorylase b for phosphorylase kinase as a function of pH. They observed that as the pH was increased from 7.6 to 8.8, the  $K_m$  of phosphorylase b decreased.  $K_m$  values could not be measured below pH 7.6. The apparent  $K_m$  of phosphorylase b for nonactivated kinase at pH 7.5 and 8.2 were  $1.25 \times 10^{-4}$  M and  $4 \times 10^{-5}$  M, respectively, assuming that a molecular weight of 250,000 was used for phosphorylase b. On the basis of a molecular weight of 100,000 for phosphorylase

these values would have to be multiplied by a factor of 2.5. The effect of activation of kinase, and hence the phosphorylation state, resulted in a lowering of the  $K_m$  of phosphorylase b at pH 7.5 and 8.2 to  $3.7 \times 10^{-5}$  M and  $1.7 \times 10^{-5}$ , respectively (based on a molecular weight of 250,000 for phosphorylase b). Activation of phosphorylase kinase did not result in a lowering of the  $K_m$  of ATP. Instead, Krebs et al., (1964) found that the constant was somewhat higher for the activated enzyme. At pH 7.5, in the presence of 10 mM  $Mg^{2+}$ , the  $K_m$  was equal to  $2.4 \times 10^{-4}$  M ATP for non-activated kinase and  $3.8 \times 10^{-4}$  M ATP for the activated enzyme. Clerck and Hujing (1972) reported a  $K_m$  of  $7 \times 10^{-5}$  M ATP for activated kinase at pH 8.2

Effectors of the kinase reaction, glycogen and  $Ca^{2+}$ , as mentioned earlier, also affect the  $K_m$  of substrate for phosphorylase kinase. Krebs et al., (1964) reported that glycogen added to the incubation mixtures decreased the  $K_m$  of phosphorylase b approximately 10-fold. Heilmeyer et al., (1970) reported that the  $K_m$  of  $Ca^{2+}$  for phosphorylase kinase was increased by a factor of 10 (from  $10^{-7}$  to  $10^{-6}$  M) in the presence of glycogen in the form of the glycogen particle. Using  $Ca^{2+}$  in the millimolar range, Krebs et al., (1959) reported that this ion is a competitive inhibitor of  $Mg^{2+}$  and a noncompetitive inhibitor of ATP in the phosphorylase kinase reaction.

A recent paper by Tessmer and Graves (1973) described the kinetic parameters of an alternative substrate, a tetradecapeptide derived from phosphorylase, containing the convertible serine residue. They obtained a  $K_m$  of  $4 \times 10^{-4}$  M peptide and  $1.6 \times 10^{-4}$  M phosphorylase b (based on 100,000 molecular weight for the monomeric form) for nonactivated phosphorylase kinase at pH 8.2. The maximal velocities obtained with peptide, however, were 4 to 9-fold lower than with phosphorylase b.

Since the normal substrate for phosphorylase kinase is a protein, control of the catalytic reaction can occur by binding of factors (e.g.,  $Ca^{2+}$ , glycogen,  $Mg^{2+}$ ,  $MgATP^{2-}$ ) to the substrate, enzyme or both (Tessmer and Graves, 1973). Therefore, an alternative substrate that does not bind these factors should be useful in studying the catalytic reaction of phosphorylase kinase. The purpose of this work is to develop a model system using an alternative substrate, a tetradecapaptide which has been recently synthesized, to do a careful study of the steady state kinetics of the phosphorylase kinase reaction. Valuable information is to be gained in comparing the kinetic parameters obtained with the synthetic substrate to those with the natural substrate, phosphorylase. Furthermore, knowing the kinetic parameters of the phosphorylase kinase reaction with the synthetic

tetradecapeptide, we can now study and compare the kinetic parameters of small and more importantly substituted synthetic peptides. From these experiments we hope to learn more about the specificity of the phosphorylase kinase reaction, and protein-protein interactions.

## EXPERIMENTAL PROCEDURE

## Materials

Peptides were synthesized on a Beckman model 990 Peptide Synthesizer, using the solid phase method described by Merrifield et al., (1966). Peptides were cleaved from the resin with hydrogen fluoride according to the procedure of Scotchler et al., (1970), and were purified to homogeneity by chromatography on Sephadex G-15 (to remove salts), on Biorex 63 (Biorad Laboratories) equilibrated in 0.2 M pyridine-acetate buffer, pH 3.1, and eluted with a linear gradient of starting buffer and 2 M pyridine-acetate buffer, pH 5.0. Purity of the peptides was determined by amino acid analysis on an updated Beckman amino acid analyzer model 120B, and by high voltage electrophoresis (HVE) in an aqueous solvent containing 2.5% v/v pyridine, 2.5% v/v glacial acetic acid, and 5% v/v n-butanol using a Camag HVE unit. Peptide spots on electropherograms were visualized after spraying with a solution containing 15 ml of 0.3% ninhydrin in 95% ethanol, 2.0 ml of collidine, and 5.0 ml of glacial acetic acid, followed by incubating the electropherogram at 90°C.

$^{32}\text{P}$ -phosphopeptide was prepared by incubating 10 mg purified peptide with 1.5 mM [ $\gamma$ - $^{32}\text{P}$ ]-ATP, 10 mM  $\text{Mg}(\text{CH}_3\text{CO}_2)_2$ , and 0.3 mg of activated phosphorylase kinase in a total volume of 2.5 ml containing 50 mM Tris-HCl, pH 7.6 and 1 mM

DTE for 2 hours at 30°C. An additional amount of 0.15 mg activated phosphorylase kinase and 1.5 mM [ $\gamma$ - $^{32}\text{P}$ ]-ATP were added and incubation continued for 2 hours at 30°C. The reaction was stopped by addition of 30% acetic acid and  $^{32}\text{P}$ -phosphopeptide was eluted with 30% acetic acid, lyophilized and desalted by passage through a Sephadex G-15 column.

Phosphorylase b was isolated from frozen rabbit skeletal muscle (Pel-Freeze Biologicals, Inc.) as described by Fischer and Krebs (1958). Residual AMP was removed by treatment with acid washed Norit A until the ratio of absorbance at 260 nm to 280 nm was 0.55 or less. When the phosphorylase was to be used in kinetic experiments it was dialyzed against 50 mM Tris HCl (pH 7.6) and 1 mM DTE.

Nonactivated phosphorylase kinase was prepared from white New Zealand rabbit back and leg muscles by the method of Brostrom et al., (1971) and stored frozen in a buffer (pH 6.8) containing 10% sucrose, 50 mM  $\beta$ -glycerophosphate and 2 mM EDTA.

[ $\gamma$ - $^{32}\text{P}$ ]-ATP was prepared essentially by the method of Glynn and Chappell (1964). Inorganic phosphate (2  $\mu\text{moles}$ ) and  $\text{NAD}^+$  (100  $\mu\text{g}$ ) were added to the reaction mixture which was added directly to a 1.5 ml AG 1-x2 (Biorad Laboratories) column, after the incubation period.

AMP, ATP, ITP, G-1-P were obtained from Sigma Chemical Company. AMPPCP, tetralithium salt, was obtained from

Boehringer-Mannheim Biochemicals. ADP, tetralithium salt, was purchased from Cal Biochem.

Glycogen was purified by the method of Anderson and Graves (1973).  $^{32}\text{P}$ -labelled phosphate (carrier free) was obtained from New England Nuclear Corporation.

Pyridine was redistilled from KOH and  $\text{KMnO}_4$  and all other chemicals were of reagent grade, and used without further purification.

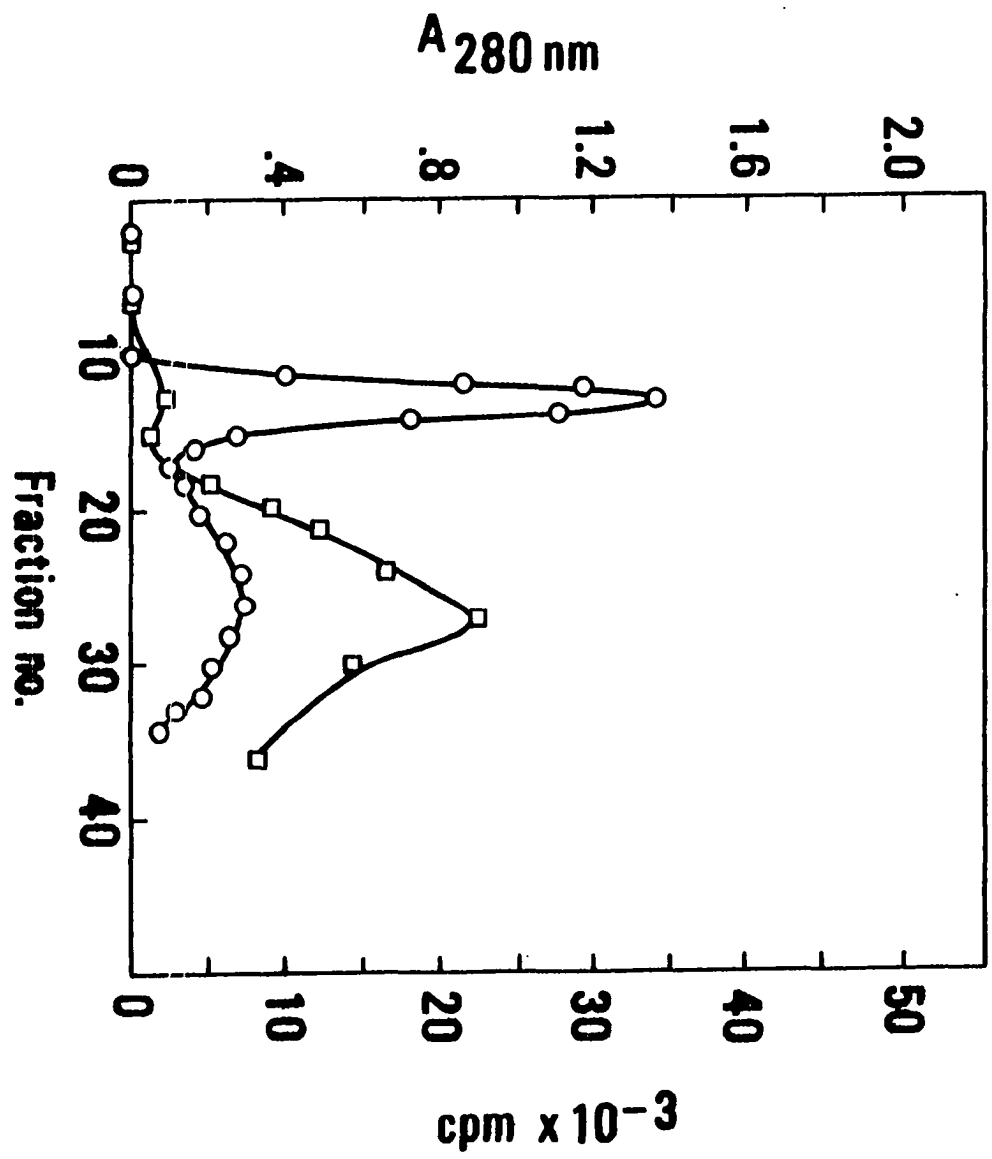
### Methods

$^{32}\text{P}$ -phosphorylated kinase was prepared by incubating it with the catalytic subunit of protein kinase in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ ,  $\text{Mg}^{2+}$  at pH 6.8. The incubation mixture contained 4 mg/ml nonactivated kinase, 1.2 mM  $[\text{}^{32}\text{P}]\text{-ATP}$ , 4 mM  $\text{Mg}(\text{CH}_3\text{CO}_2)_2$ , 1 mM DTE, and 50 mM  $\beta$ -glycerophosphate, pH 6.8, in a total volume of 2.5 ml. The reaction mixture was incubated for 40 min at 30°C. Excess  $[\text{}^{32}\text{P}]\text{-ATP}$  and other low molecular weight materials were removed by passing the activated kinase over a Sephadex G-25 column (1.4 cm x 50 cm), equilibrated with a buffer (pH 6.8) containing 10% sucrose, 50 mM  $\beta$ -glycerophosphate and 2 mM EDTA (Figure 1). The catalytic subunit of a protein kinase was isolated by the procedure of Beavo et al., (1974) and supplied by J. R. Skuster.

Sodium dodecyl sulfate (SDS) polyacrylamide gel



Figure 1. Activation and chromatography of phosphorylase kinase. Nonactivated phosphorylase kinase (4 mg/ml) was activated with the catalytic subunit of cyclic AMP-dependent protein kinase (1 to 1500 by weight),  $\gamma$ - $^{32}\text{P}$ ATP, (1.2 mM,  $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)$  (4 mM), DTE (1 mM,  $\beta$ -glycero-P pH 6.8 (50 mM), EDTA (2 mM), EGTA (0.1 mM). Incubation was for 40 min at 30°C. Activated phosphorylase kinase was chromatographed on Sephadex G-25 (coarse, 1.5 x 40 cm) and eluted with sucrose (10%),  $\beta$ -glycero-P (50 mM), EDTA, (2 mM), pH 6.8. (O absorbance at 280 nm,  $\square$   $^{32}\text{P}$  in cpm x  $10^{-3}$ )



electrophoresis of [ $^{32}\text{P}$ ]-activated phosphorylase kinase was carried out as described by Hayakawa et al., (1973). The denaturation mixture contained 100  $\mu\text{l}$  kinase (1-2 mg/ml), 20  $\mu\text{l}$  SDS (6%), and 20  $\mu\text{l}$   $\beta$ -mercaptoethanol (6%). The mixture was heated for 30 min at 60°C. After cooling, 40  $\mu\text{l}$  glycerol and 20  $\mu\text{l}$  bromphenol blue (0.03%) were added, and 25  $\mu\text{l}$  portions (12.5 - 15  $\mu\text{g}$  protein) were applied to the gels (0.6 cm x 8 cm). Gels contained 7.5% acrylamide monomer and 0.13% bis-acrylamide. The gel buffer contained 100 g Tris, 10 g EDTA, 3.8 g boric acid, and 1 g SDS per 500 ml (pH 9.3). The gel buffer diluted 1:1 with water was used as the running buffer. Electrophoresis was performed at 8 ma/gel. Gels were stained overnight in a solution containing 0.1% Coomassie blue R250, 50% methanol, 9.2% acetic acid and were destained electrophoretically in 7.5% acetic acid, in a Canalco Quick Destain unit (Canalco Company).

To determine [ $^{32}\text{P}$ ] incorporated per subunit of kinase, the protein bands were removed and placed in scintillation vials. After addition of 200  $\mu\text{l}$  30%  $\text{H}_2\text{O}_2$ , the gels were digested for 2 hrs at 60-65°C. The radioactivity was determined in a Packard Tricarb scintillation spectrometer after solubilizing the digested gel slices in 10 ml Aquasol (New England Nuclear Corporation).

The extent of phosphorylation of phosphorylase kinase and phosphorylase were calculated by assuming molecular

weights of  $1.28 \times 10^6$  (Cohen, 1973) and  $1 \times 10^5$  (Cohen et al., 1971), respectively.

Phosphorylase kinase was assayed either by measuring the increase in phosphorylase a activity as described by Brostrom (1971) or by determining [ $^{32}\text{P}$ ] incorporation into phosphorylase from [ $\gamma$ - $^{32}\text{P}$ ]-ATP using the filterpaper assay described by Reimann et al., (1971).

Phosphorylase was assayed in the direction of glycogen synthesis according to the method of Illingworth and Cori (1953).

Formation of phosphorylated peptide was measured according to a method developed by G. W. Tessmer. Reaction aliquots, 10 or 20  $\mu\text{l}$ , were applied to 1 cm x 2 cm strips of Whatman P81 phosphocellulose paper, which binds only the phosphorylated peptide. The reaction was stopped by placing the paper strips in 1 N acetic acid. The papers were washed 3 to 4 times at 20 min each with 1 N acetic acid to remove excess [ $^{32}\text{P}$ ]-ATP. Ten ml of the acid was allowed per paper strip. The papers were then rinsed with ethanol, followed by ether, dried, and counted in 15 ml of Bray's scintillation solution containing 60 g of naphthalene, 4 g of 2.5-diphenyloxazole (PPO), 0.4 g of P-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), 100 ml methanol, 20 ml of ethylene glycol and made to 1 l with 1,4-dioxane.

Formation of [ $^{32}\text{P}$ ]-phosphorylated peptide which did not

bind to P81 phosphocellulose paper strips was measured by using a column method as follows. The reaction mixture (60  $\mu$ l) was stopped by an addition of 0.5 ml of 30% acetic acid. The total mixture was then applied to a 6.5 cm column of Ag 1-x8 ion exchange resin (BioRad Laboratories) in a disposable pasteur pipette. The ion exchange resin bound all the [ $\gamma^{32}\text{P}$ ]-ATP while the phosphorylated peptide was eluted with 9.5 ml of 30% acetic acid directly into a scintillation vial. Radioactivity was determined in a Packard TriCarb scintillation spectrometer by measuring Cerenkov radiation in the tritium channel as described by Ross (1969).

## RESULTS

This work describes the use of a synthetic peptide as an alternative substrate to study the kinetic mechanism of phosphorylase kinase. Another interesting question to be answered is what constitutes a good minimal peptide for phosphorylase kinase. In other words, which residue(s) in a good minimal peptide substrate is essential for catalysis to take place? This work will also attempt to provide some insight on the question of kinase specificity.

### Validity of the Model System

In order to use an alternative substrate, one should demonstrate that this substrate serves as a good model for the study of the catalytic reaction of the enzyme in question, as compared to the enzyme's natural substrate. It had already been shown by Tessmer and Graves (1973) that the correct seryl residue was phosphorylated, i.e., the serine residue located between isoleucine and valine and not the N-terminal serine of the peptide substrate. Some aspects of the kinase reaction using the tetradecapeptide as a model system have been reported (Carlson et al., 1975) and detailed results of such experiments are described below. Features of the kinase reaction studied in the model system are pH dependence of activity, lag in the rate of product formation, effect of preincubation and effect of kinase concentration on the rate

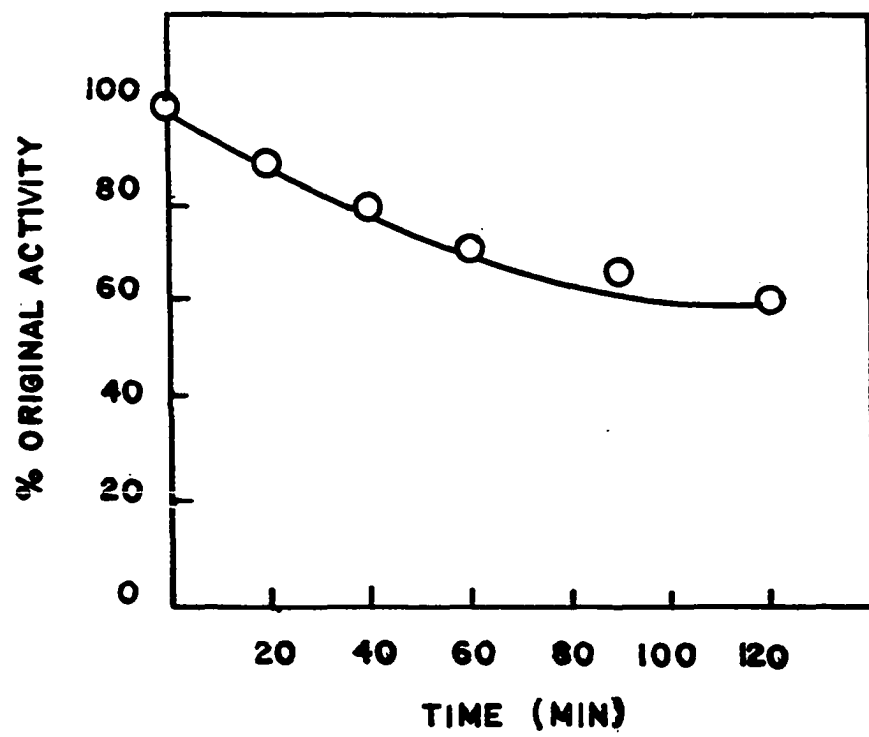
of product formation.

### Choosing the assay buffer

Hepes buffer has been often the buffer of choice in enzyme work since it has a pK around neutrality, and secondly, it does not complex metal ions (Good et al., 1966). However, kinetic experiments with phosphorylase kinase in this buffer very often were not reproducible. Stability of kinase was therefore tested as a function of time in this buffer. Figure 2 shows that after 2 hours of incubation 40% of the kinase activity has been lost. Inclusion of 10% sucrose did lower the rate of inactivation but not the extent of loss of activity. Phosphorylase kinase activity, however, is stable in Tris- $\beta$ -glycerophosphate buffer. Because of the fact that this buffer binds metal ions, it was decided not to use this buffer for kinetic experiments. Activity of phosphorylase kinase, measured in terms of peptide conversion, was compared in several buffers. Two of these buffers, MOPS and PIPES (Good et al., 1966) were tested at pH 6.8. Peptide conversion was too low to be of any use in kinetic experiments, in which substrates are varied over a 10-fold concentration range. Activity of kinase in Tris- $\beta$ -glycerophosphate was also compared in Tris and Hepes buffers. Assigning a value of 100% to the activity level measured in Tris- $\beta$ -glycerophosphate pH 7.7, activities measured in Tris and Hepes at

Figure 2. Stability of phosphorylase kinase in HEPES buffer. Activated phosphorylase kinase (30  $\mu\text{g/ml}$ ) is incubated at  $0^{\circ}\text{C}$  in the presence of 4 mM HEPES, 1 mM DTE, pH 6.8. At the times indicated 10  $\lambda$  aliquots are removed and activity is measured by the extent of peptide converted in 5 min under the following assay conditions: ATP (1.5 mM),  $\text{MgCl}_2$  (6 mM), tetradeca peptide (1 mM), HEPES pH 7.6 (20 mM), DTE (1 mM), phosphorylase kinase final concentration, (5  $\mu\text{g/ml}$ )





pH 7.7 were 109% and 147%, respectively. It is interesting to note that kinase exhibits 50% more activity in HEPES than in Tris- $\beta$ -glycerophosphate (or, conversely, 50% inhibition in Tris- $\beta$ -glycerophosphate buffer). Since phosphorylase kinase activity was found to be stable in Tris buffer at pH 7.7 (not shown), this buffer was chosen for the kinetic experiments, while the groundwork for the model system was done using HEPES buffer.

#### pH dependence of activity

One of the characteristic properties of phosphorylase kinase is its activity at pH 6.8 and pH 8.2. In fact, the ratio of activities at pH 6.8 to 8.2 is widely used as an indication of the state of activation of phosphorylase kinase (Cohen, 1973; Krebs et al., 1964). Krebs et al., (1959,1964) have shown that a plot of kinase activity versus pH with its natural substrate phosphorylase, is characteristically S-shaped. Figures 3 and 4 show the pH profile of nonactivated kinase and activated kinase, respectively. The curves for both forms of kinase show the similar S-shaped curves using the synthetic peptide as substrate. Initial work on the pH dependence of kinase activity described by Fischer and Krebs (1955), indicated that the pH optimum of kinase differed markedly depending on the buffer employed. The experiment shown in Figure 4 with three different buffers, Tris, Tes and

Figure 3. pH profile of nonactivated phosphorylase kinase. Tetradecapeptide conversion is plotted as a function of pH, under the following assay conditions: 0.6 mM tetradecapeptide, 8 mM  $^{32}\text{P}$ -ATP, 27 mM  $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$ , 40 mM HEPES, 10 mM mercaptoethanol and 1.7  $\mu\text{g}/\text{ml}$  non-activated phosphorylase kinase.  $^{32}\text{P}$  incorporation into peptide was measured after 5 min of incubation at 30°C, as described under Methods

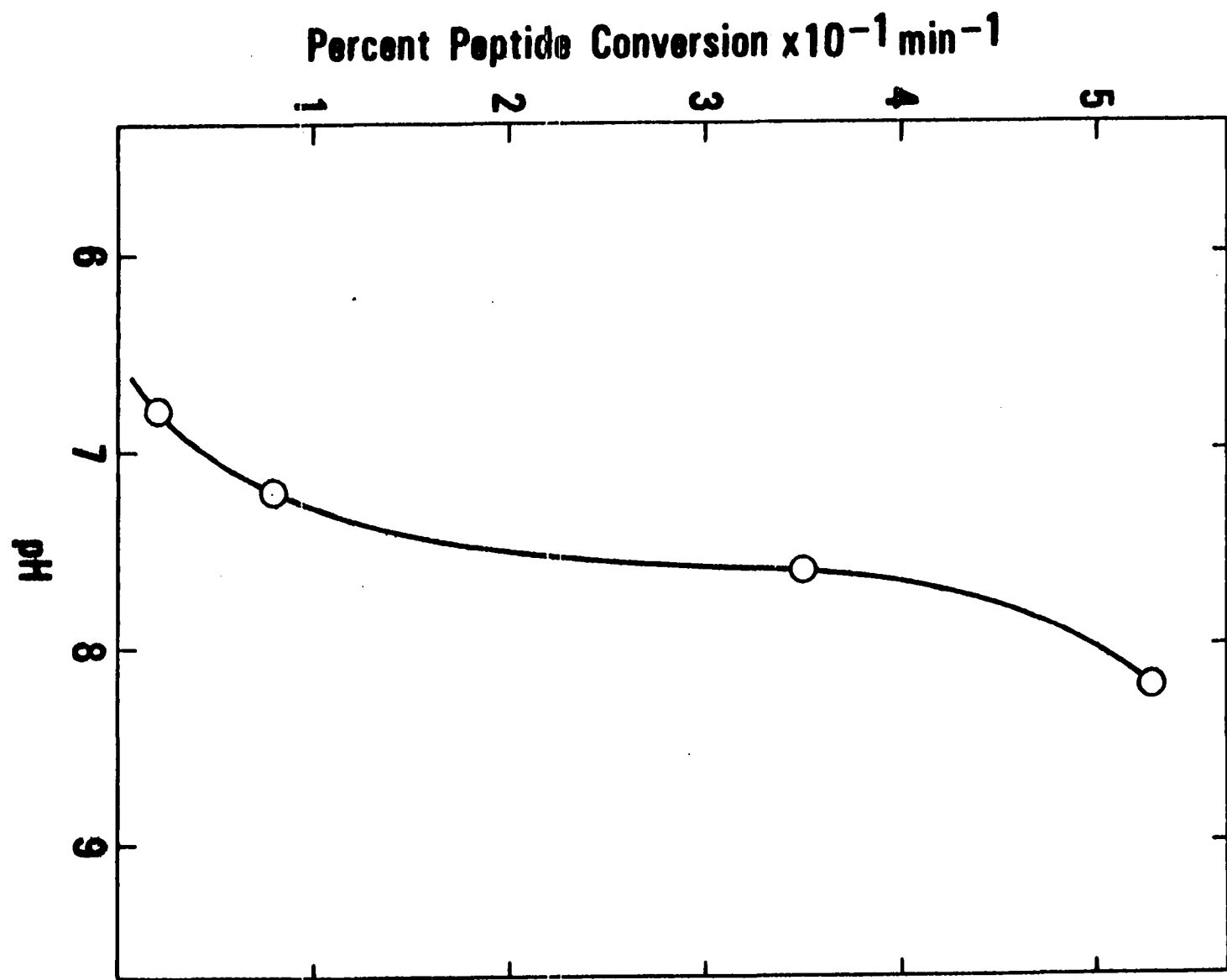
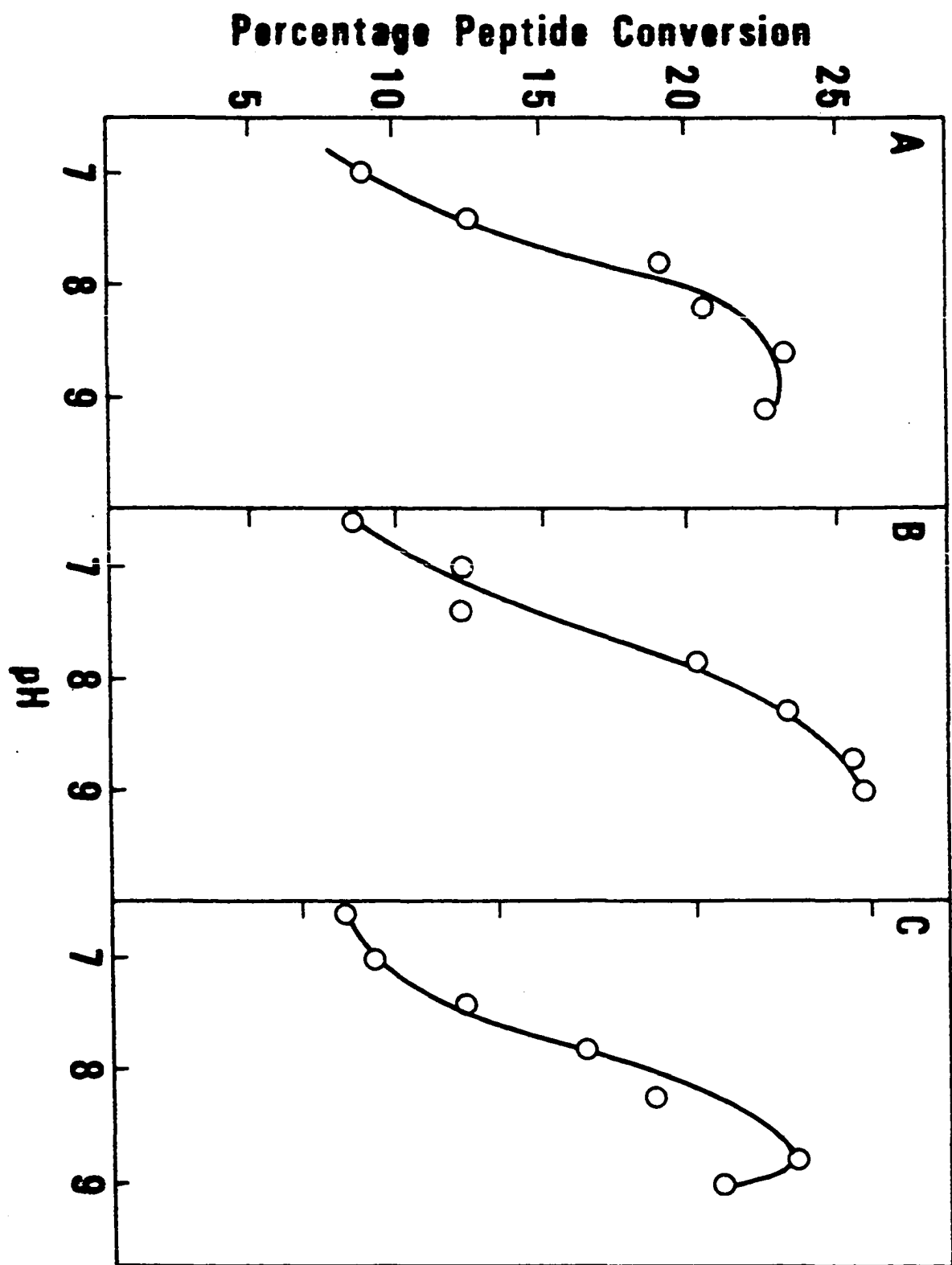


Figure 4. pH profiles of activated phosphorylase kinase in various buffers. Tetradecapeptide conversion is plotted as a function of pH in Tris (panel A), TES (panel B), and Tris- $\beta$ -glycerophosphate (panel C). The assay contained 0.8 mM tetradecapeptide, 0.75 mM  $^{32}\text{P}$ -ATP, 11 mM  $\text{MgCl}_2$ , 3.7  $\mu\text{g/ml}$  of activated kinase, 1 mM DTE, and 50 mM Tris (A) or 50 mM TES (B), or 21 mM Tris-21 mM  $\beta$ -glycerophosphate (C).  $^{32}\text{P}$  incorporation into peptide was measured after 5 min of incubation at 30°C as described under Methods



the conventionally used combination of Tris- $\beta$ -glycerophosphate indicated no marked differences. It should be kept in mind though, that Tes has no significant buffering capacity at pH 8.5 and above as its  $pK = 7.5$ . The same is true for Tris below pH 7.3 ( $pK = 8.3$ ). From the data shown in Figures 3 and 4 the ratio of activities at pH 6.8/8.2 can be calculated; these are 0.03 and 0.4 for nonactivated and activated phosphorylase kinase, respectively. With phosphorylase as substrate using the indirect assay for phosphorylase kinase (Brostrom et al., 1971; Illingworth and Cori, 1953), these values are 0.02 and 0.36, respectively.

#### Lag in the catalytic reaction

A second property of nonactivated phosphorylase kinase reaction with phosphorylase as substrate is the lag in the rate of product formation (Kim and Graves, 1973). A similar lag in the catalytic reaction is noted with the synthetic tetradecapeptide as substrate as is shown in Figure 5 (closed circles). The lag is completely abolished or diminished upon preincubation of nonactivated phosphorylase kinase with peptide substrate, as shown in Figure 5 (open circles). Similar results were obtained by Kim and Graves (1973) using phosphorylase b as substrate.

The lag in the catalytic reaction also diminishes in the presence of the peptide substrate (Figure 6). A similar

Figure 5. Effect of preincubation on the lag in the catalytic reaction. The rate of formation of  $^{32}\text{P}$ -peptide was measured without preincubation or after a 15 min preincubation period of phosphorylase kinase in the presence of peptide. The incubation mixture contained 0.6 mM tetradecapeptide, 0.1 mM  $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ , 40 mM HEPES pH 6.8, 10 mM metcaptoethanol, 2.7  $\mu\text{g}/\text{ml}$  of nonactivated phosphorylase kinase and 8 mM  $^{32}\text{P}$ -ATP, 20 mM  $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$ . The reaction was initiated with the addition of  $^{32}\text{P}$ -ATP. ● No preincubation, ○ 15 min preincubation



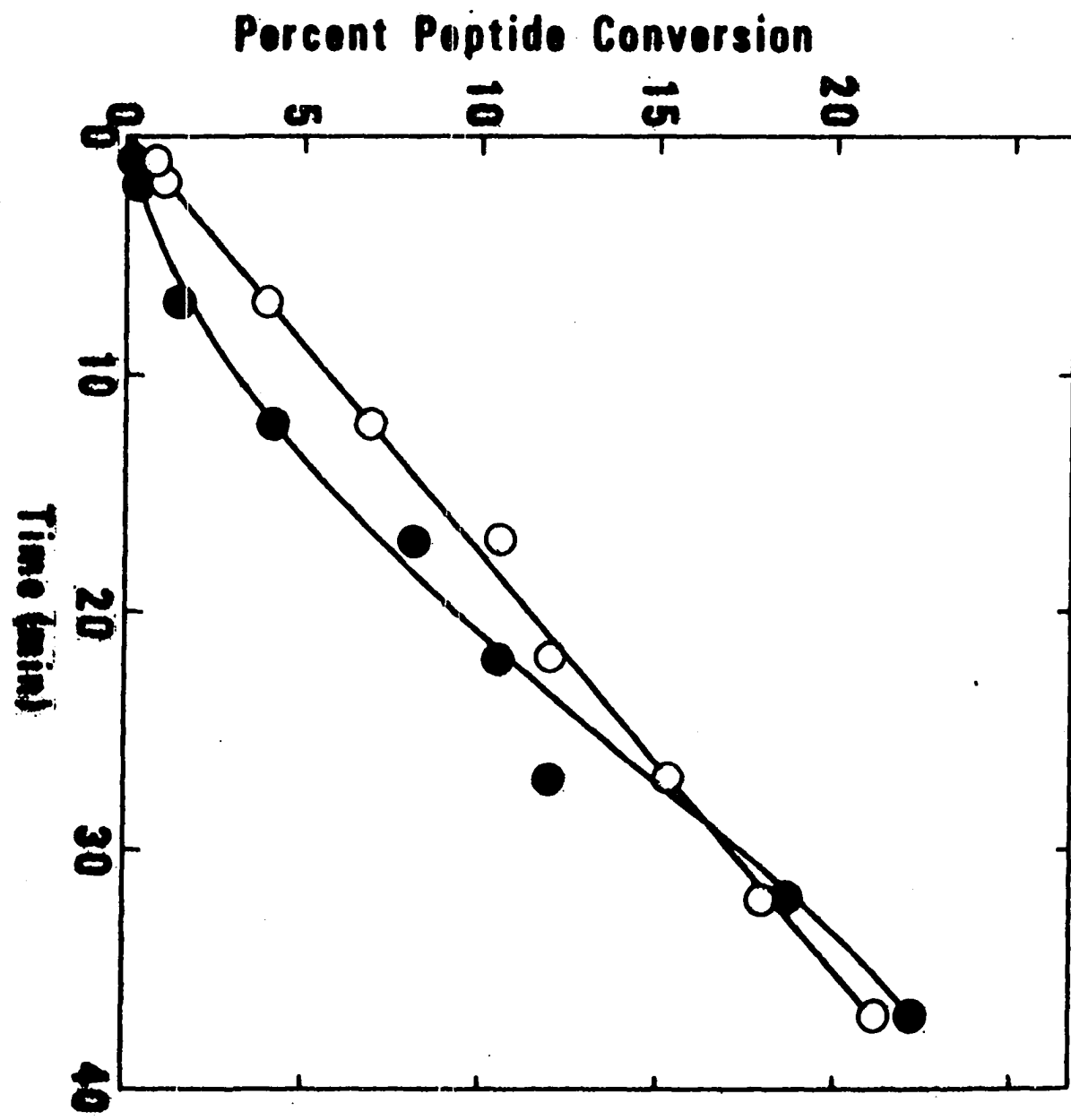
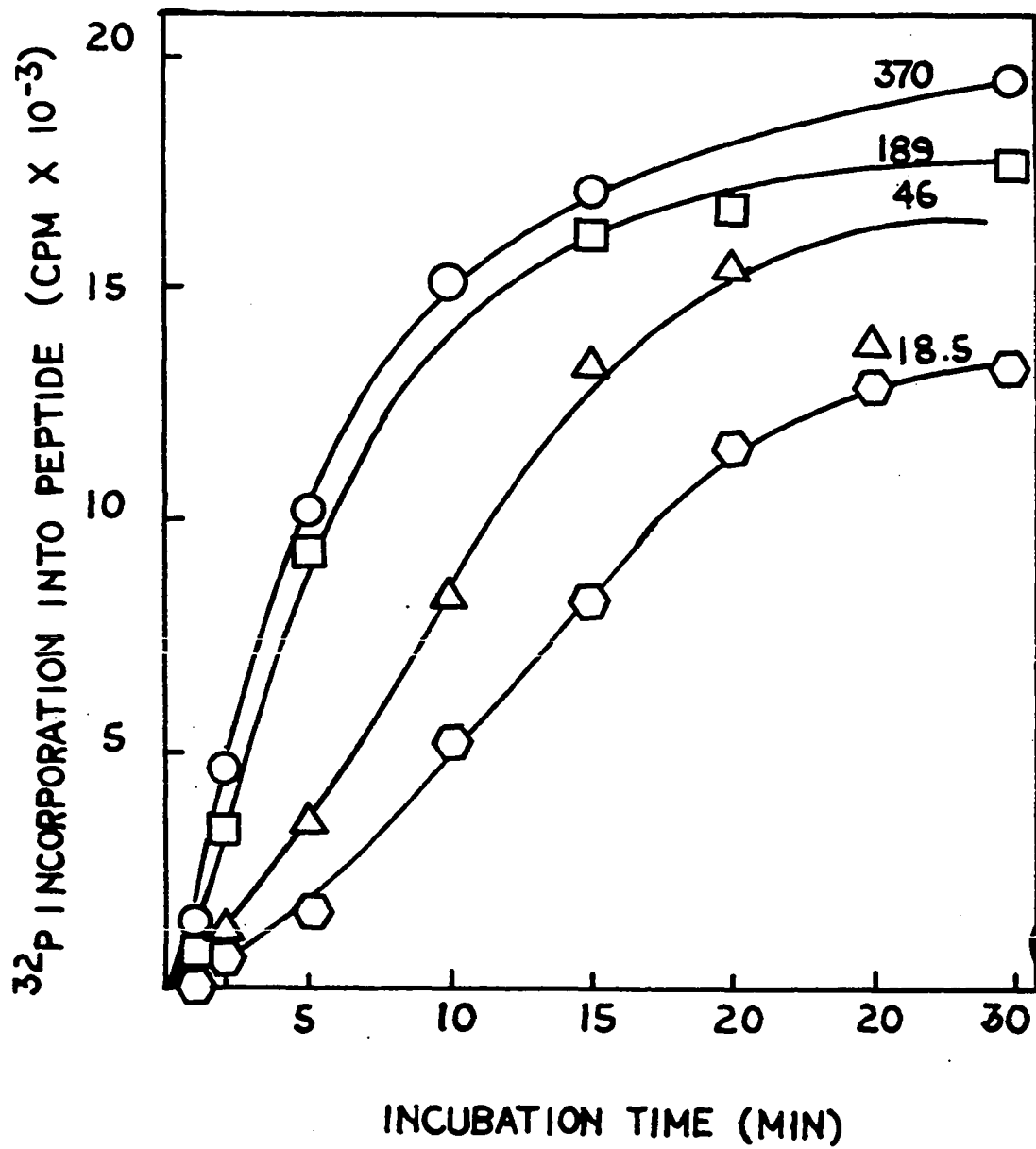


Figure 6. Effect of phosphorylase kinase concentration on the lag in the catalytic reaction. The incubation mixtures contained 0.6 mM tetradecapeptide, 40 mM HEPES-10 mM mercaptoethanol, 8 mM  $^{32}\text{P}$ -ATP, 20 mM  $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$ , and phosphorylase kinase concentrations in  $\mu\text{g/ml}$  as indicated on the graph



experiment was performed by DeLange et al., (1968) with identical results except that phosphorylase b was used as the substrate.

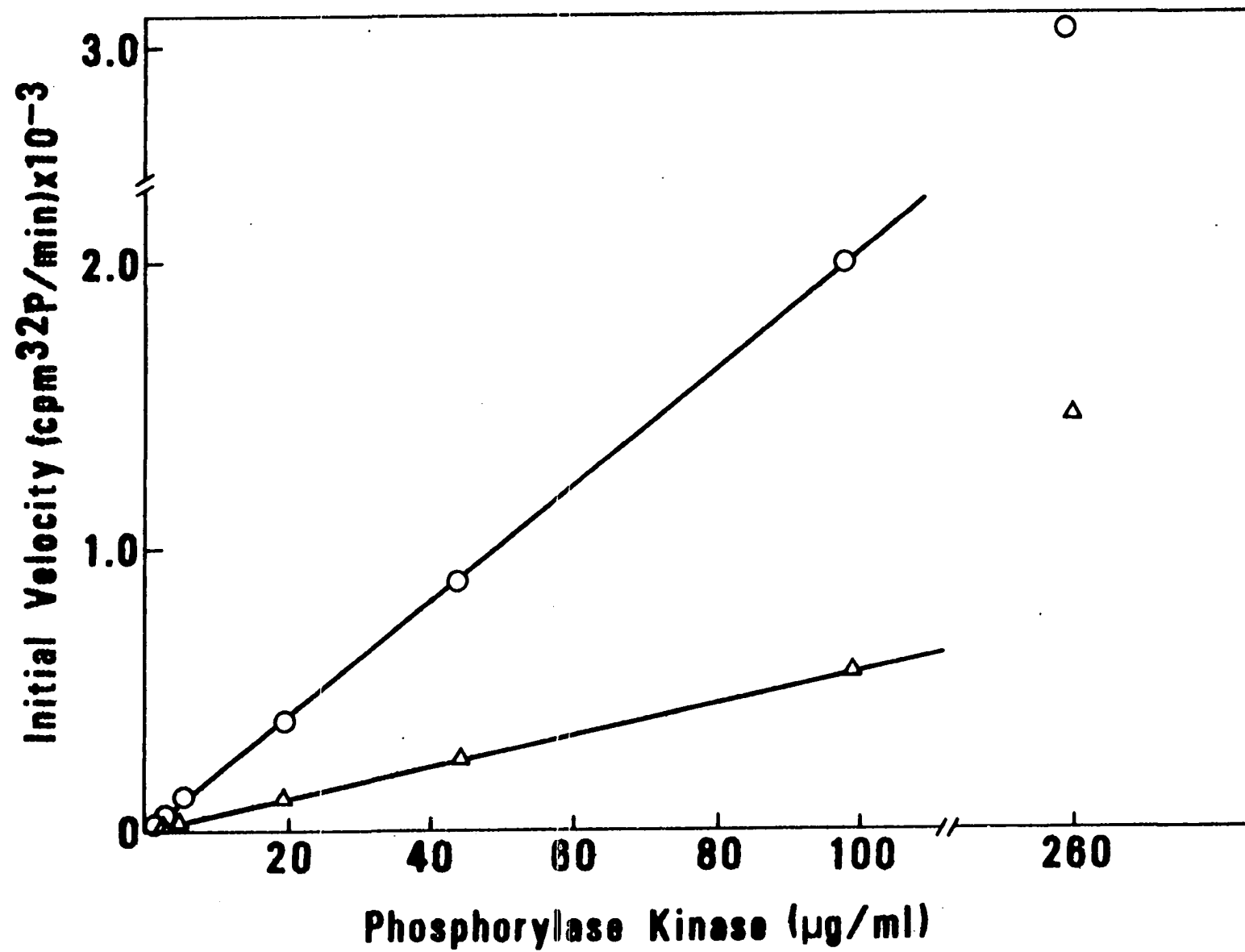
Thus it seems that in every case discussed, the peptide substrate can substitute for its natural counterpart, phosphorylase, which itself is almost two orders of magnitude larger in size than the synthetic peptide.

#### Consideration of Kinetic Parameters

##### Linearity of rate of product formation

The first condition that must be satisfied before kinetic studies can be initiated is that the rate of product formation must vary in a linear fashion with time. The progress curve of nonactivated phosphorylase kinase is, however, not linear (Figure 5, closed circles). Preincubation of kinase with one of the substrates, peptide in this case, for 10-15 minutes and in the presence of  $\text{Ca}^{2+}$  does result in a linear progress curve (Figure 5, open circles). However, the enzyme itself has changed during preincubation. The increase in activity was in the order of 3 to 3.5-fold. This result is shown in Figure 7. Initial velocities were plotted versus enzyme concentration. The open circles show the initial velocities that were measured after a preincubation period of 15 minutes. Each point represents the slope of a progress curve. The curve represented by the triangles was obtained

Figure 7. Plot of initial velocity versus nonactivated phosphorylase kinase concentration. Nonactivated phosphorylase kinase was assayed with and without preincubation in the presence of 0.6 mM tetradecapeptide, 40 mM HEPES-10 mM mercaptoethanol, 0.1 mM  $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ , 8 mM  $^{32}\text{P}$ -ATP, 20 mM  $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)_2$ . Initial velocities were calculated from the progress curves.  $\Delta$  no preincubation,  $\circ$  15 min preincubation



by measuring the initial velocity at a time point close to  $t=0$ . This is accomplished by drawing a tangent to the progress curve at  $t=0$ , and by calculating the slope of this tangent. The same increase in specific activity of phosphorylase kinase was also measured after the preincubation period by diluting the kinase 10-fold with cold buffer, followed by the indirect assay for kinase activity.

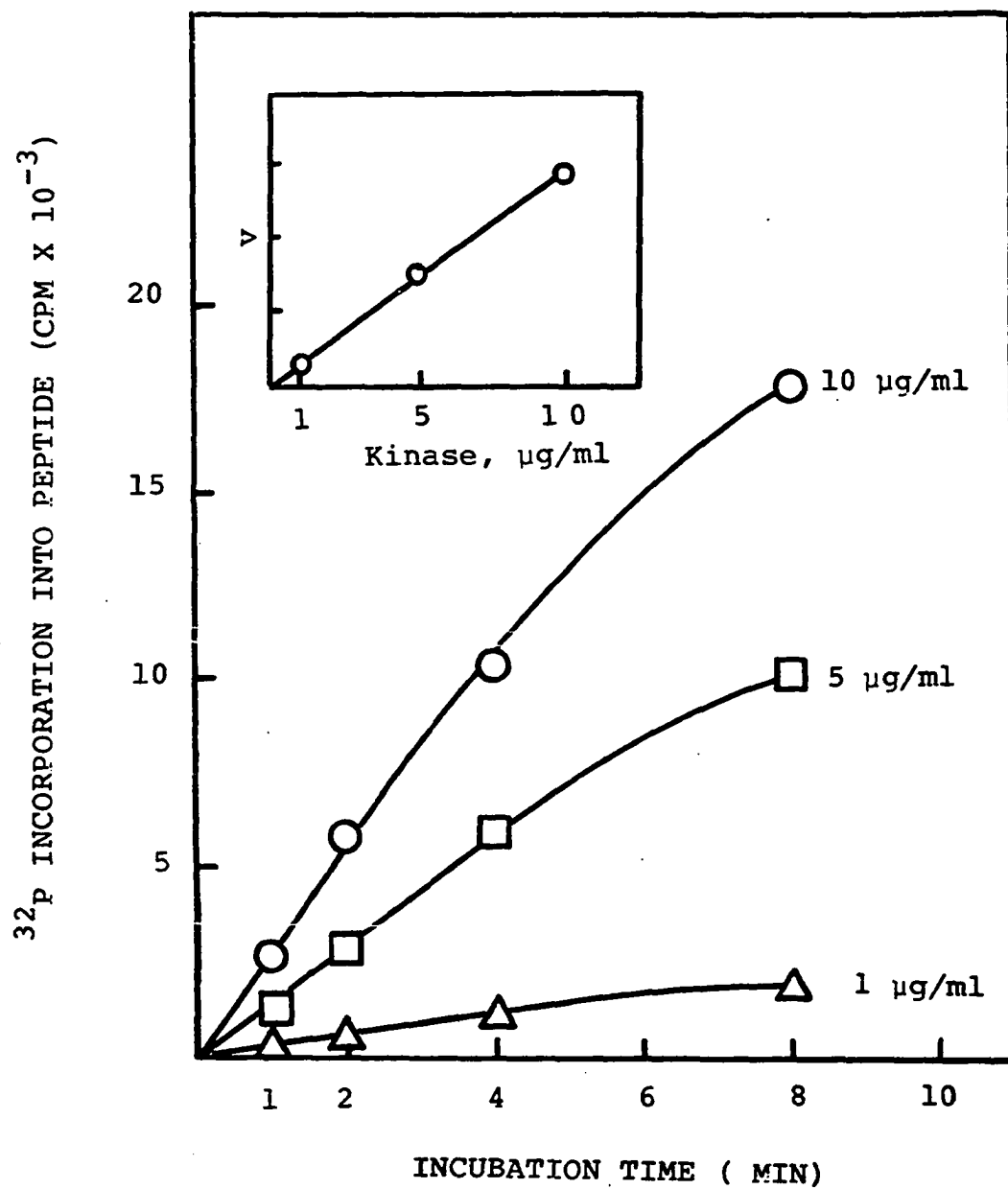
Although linear progress curves are obtained with preincubated, nonactivated kinase, this preincubation procedure would complicate the already complex experiments of initial rate studies. Fortunately, another form of phosphorylase kinase, activated in the presence of cyclic-AMP-dependent protein kinase, shows linear progress curves (Figure 8). Furthermore, phosphorylase kinase activated in this manner and in the presence of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  can be fully characterized by its pH 6.8/8.2 ratio and the moles of phosphate incorporated per mole of subunit. In addition, this form of kinase can also be stored frozen, but only for up to one month, without losing activity.

#### Initial velocity versus enzyme concentration

An additional criterion which must be satisfied before kinetic studies can be performed is the fact that the initial velocity must be proportional to enzyme concentration, this is implicit in the Michaelis-Menten equation. The plot of

Figure 8. Linearity of the activated phosphorylase kinase reaction. Activated phosphorylase kinase was assayed in the presence of 0.8 mM tetradeca peptide, 50 mM Tris HCl pH 7.6, 1 mM DTE, 1.5 mM  $^{32}\text{P}$ -ATP, 11.5 mM  $\text{MgCl}_2$ , at the concentrations indicated. Inset shows a plot of initial velocity versus enzyme concentration





initial velocity versus enzyme concentration is shown on the inset in Figure 7 for nonactivated kinase and in Figure 8 for activated kinase.

In all subsequent initial rate studies activated kinase will be used.

#### Kinetic mechanism from initial rate studies

Preliminary experiments of initial rate studies were done using 2 mM free  $Mg^{2+}$  in 20 mM HEPES buffer, pH 7.6 (see Appendix A for calculations of free  $Mg^{2+}$  levels). In subsequent experiments, 50 mM Tris pH 7.6 buffer was substituted for HEPES as the stability of phosphorylase kinase appeared to be adversely affected by the latter or by impurities present in this buffer. No such problems were encountered using Tris buffer.

Plots of the reciprocal of initial velocity versus the reciprocal of substrate concentration, at constant but various levels of the other substrate are shown in Figure 9 and 10. It is evident from these plots that the lines converge to a common intersection point in the upper left hand quadrant. The convergence indicates that the Ping Pong mechanism can be excluded and that the mechanism is one of the Sequential type. In addition, the linear nature of the curves indicate that the covalently modified form of phosphorylase kinase obeys Michaelis-Menten type kinetics.

Figure 9. Double reciprocal plot of initial velocity versus peptide concentration at constant MgATP concentrations. The reciprocal of the initial velocities at several but constant levels of MgATP were plotted as a function of the reciprocal of peptide concentration. The incubation mixtures contained 4.2  $\mu\text{g/ml}$  of activated phosphorylase kinase, 50 mM Tris·HCl pH 7.6, 1 mM DTE, and 2 mM free  $\text{Mg}^{2+}$  in addition to  $\text{Mg}^{32}\text{P}$ -ATP and tetradecapeptide. MgATP concentrations were:  $\square$  0.19 mM,  $\blacktriangle$  0.25 mM,  $\triangle$  0.37 mM,  $\bullet$  0.75 mM

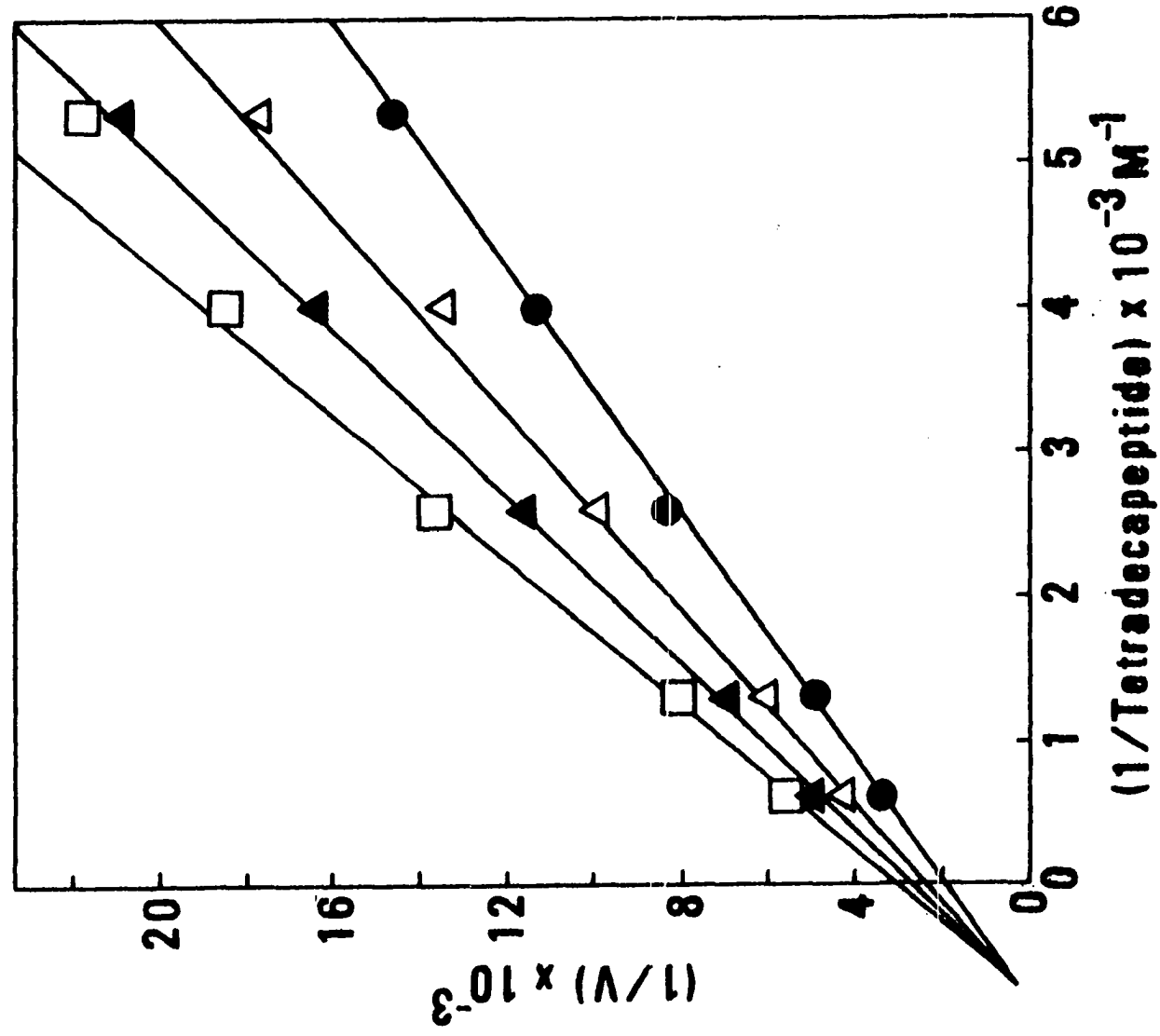
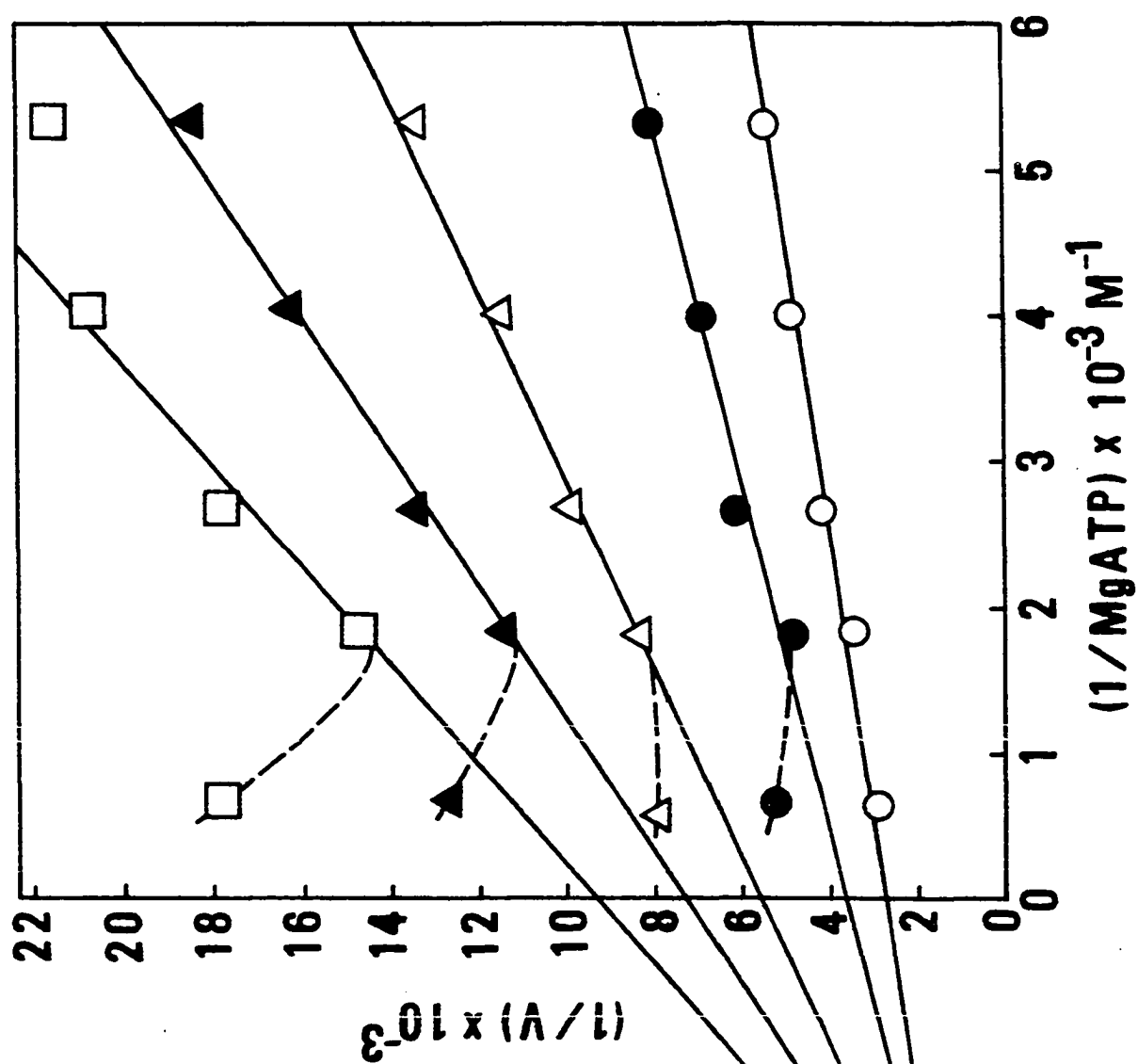


Figure 10. Double reciprocal plot of initial velocity versus MgATP concentration at constant tetradecapeptide concentrations. The reciprocal of the initial velocities at several but constant levels of tetradecapeptide concentrations were plotted as a function of the reciprocal of MgATP concentration. The incubation mixtures contained 4.2  $\mu\text{g/ml}$  of activated phosphorylase kinase, 50 mM Tris·HCl pH 7.6, 1 mM DTE, and 2 mM free  $\text{Mg}^{2+}$  in addition to tetradecapeptide and MgATP. Tetradecapeptide concentrations were:  $\square$  0.19 mM,  $\blacktriangle$  0.25 mM,  $\triangle$  0.37 mM,  $\bullet$  0.75 mM,  $\circ$  1.5 mM



The secondary plots constructed from slopes and intercepts of the primary double reciprocal plots are shown in Figures 11 and 12. Figures 11 and 12 show the secondary plots of intercepts and slopes respectively, using the synthetic peptide as substrate. From the secondary plots the kinetic parameters for the phosphorylase kinase reaction can be obtained. The intersection points of the lines with the abscissa on the intercept plots give the Michaelis constants,  $K_a$  and  $K_b$  of the substrates at infinite concentration of the other substrate. Maximal velocity in the forward direction,  $V_1$ , at infinite concentration of both substrates is obtained at the common intersection point on the ordinate of the intercept plot. From the slope plots the same parameters are obtained.

Figures 13 and 14 show the double reciprocal plots with phosphorylase b, rather than the synthetic peptide as substrate. Similar converging line patterns are obtained, with the intersection point also in the upper left-hand quadrant. Thus also with the protein substrate normal Michaelis-Menten type kinetics are observed for activated phosphorylase kinase.

The upward curvature at low reciprocal  $\text{MgATP}^{2-}$  levels is most likely due to  $\mu\text{molar}$  amounts of free ATP still present. This curvature is more pronounced when phosphorylase b is used as substrate. Phosphorylase b is known to bind  $\text{Mg}^{2+}$  and may therefore act as a Mg sink, lowering the free  $\text{Mg}^{2+}$  level and

Figure 11. Intercept plot. Intercepts with the  $1/v$  axis of Figures 9 and 10 are plotted as a function of the reciprocal MgATP and tetradecapeptide concentrations, respectively.  
○ Intercepts from Figure 9. ● Intercepts from Figure 10



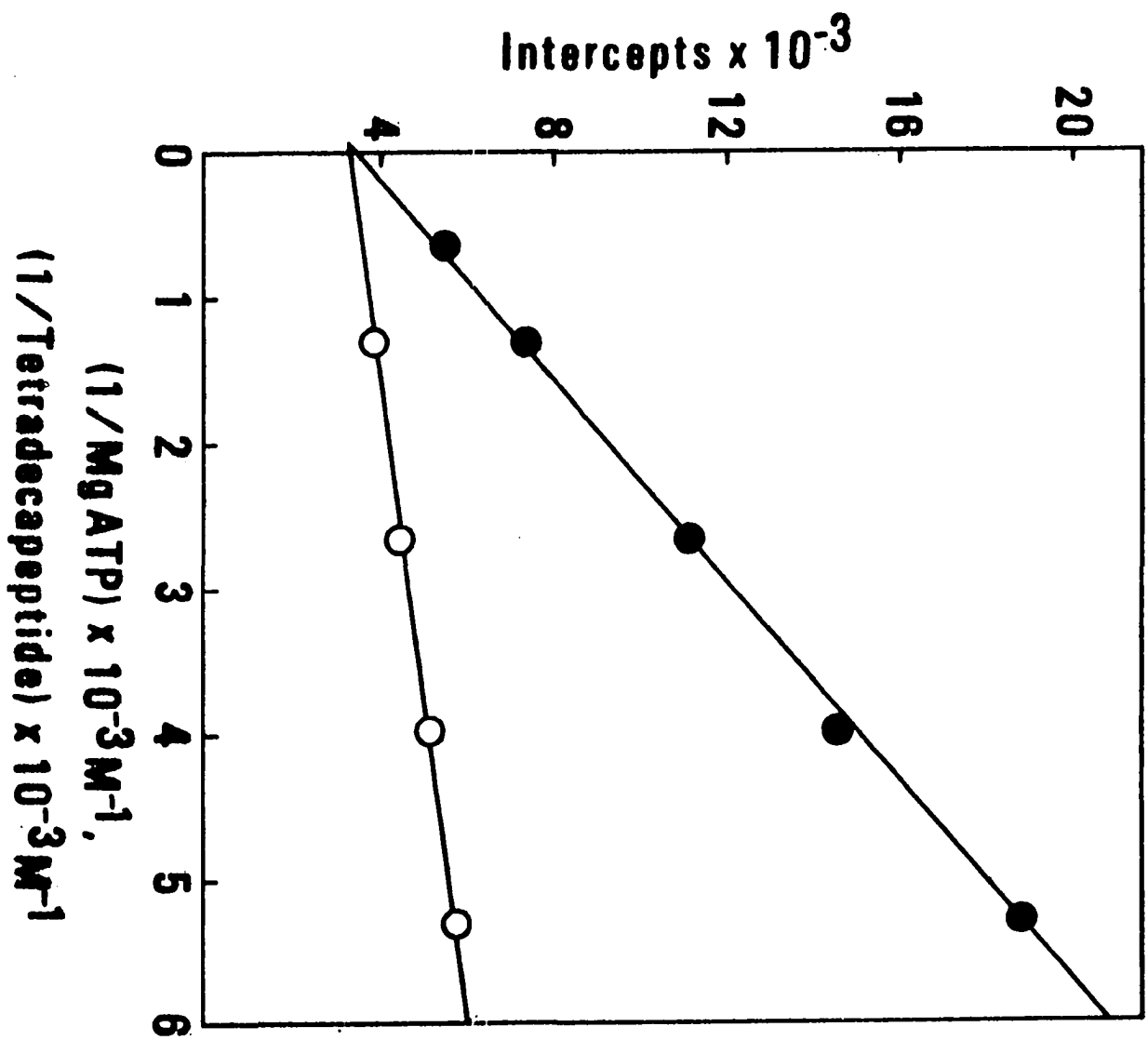


Figure 12. Slope plot. Slopes of the family of curves of Figures 9 and 10 are plotted versus the reciprocal of MgATP and tetradecapeptide concentrations, respectively. ○ Slopes calculated from Figure 9, ● slopes calculated from Figure 10

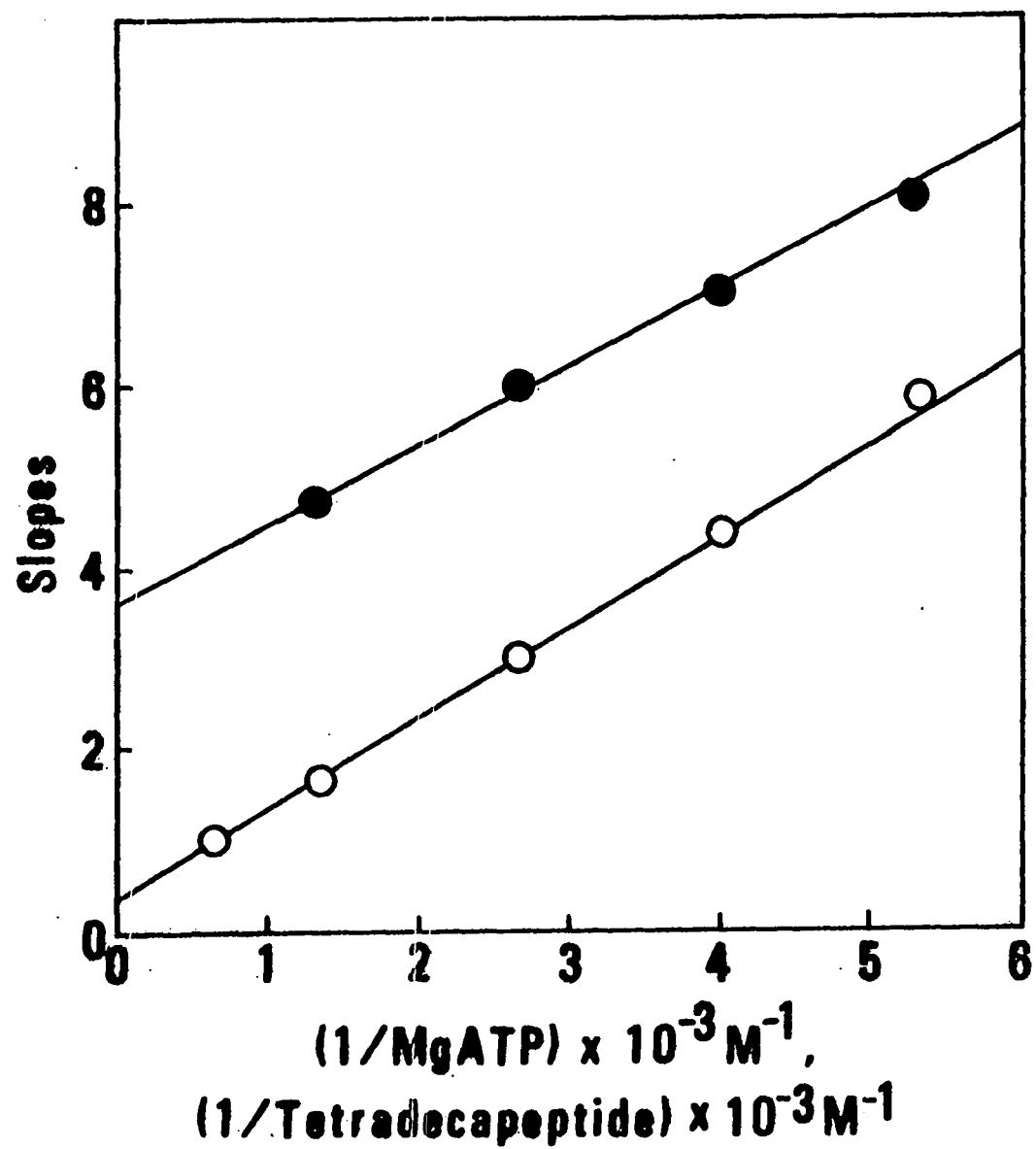


Figure 13. Double reciprocal plot of initial velocity versus phosphorylase b concentration at constant MgATP concentrations. The reciprocal of the initial velocities at several but constant levels of MgATP were plotted as a function of the reciprocal of phosphorylase b concentration. The incubation mixture contained 0.38  $\mu\text{g/ml}$  of activated phosphorylase kinase, 50 mM Tris·HCl pH 7.6, 1 mM DTE, and 2 mM free  $\text{Mg}^{2+}$  in addition to  $\text{Mg}^{32}\text{P}$ -ATP and phosphorylase b. MgATP concentrations were:  $\square$  0.19 mM,  $\overline{\Delta}$  0.25 mM,  $\Delta$  0.375 mM

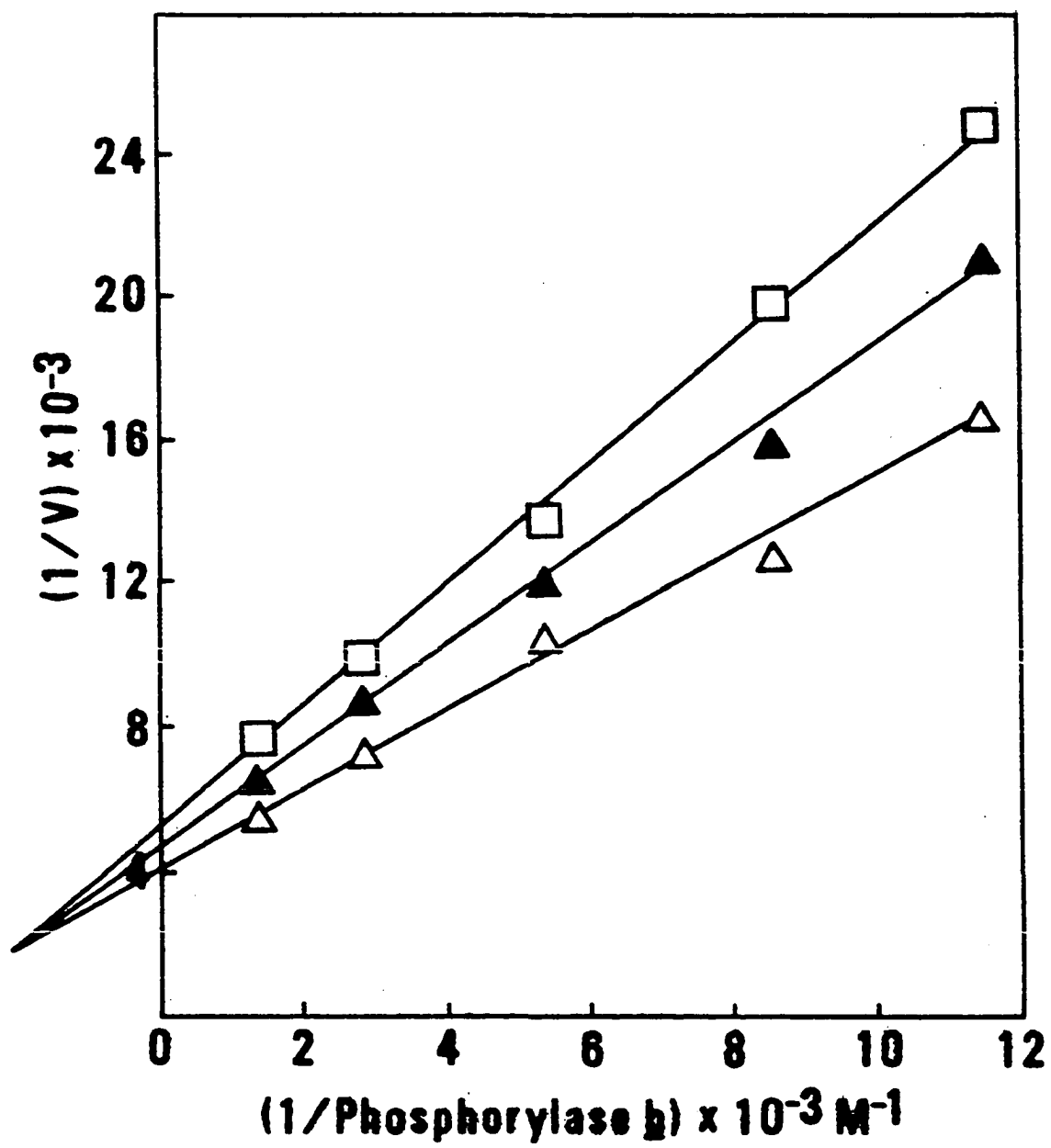
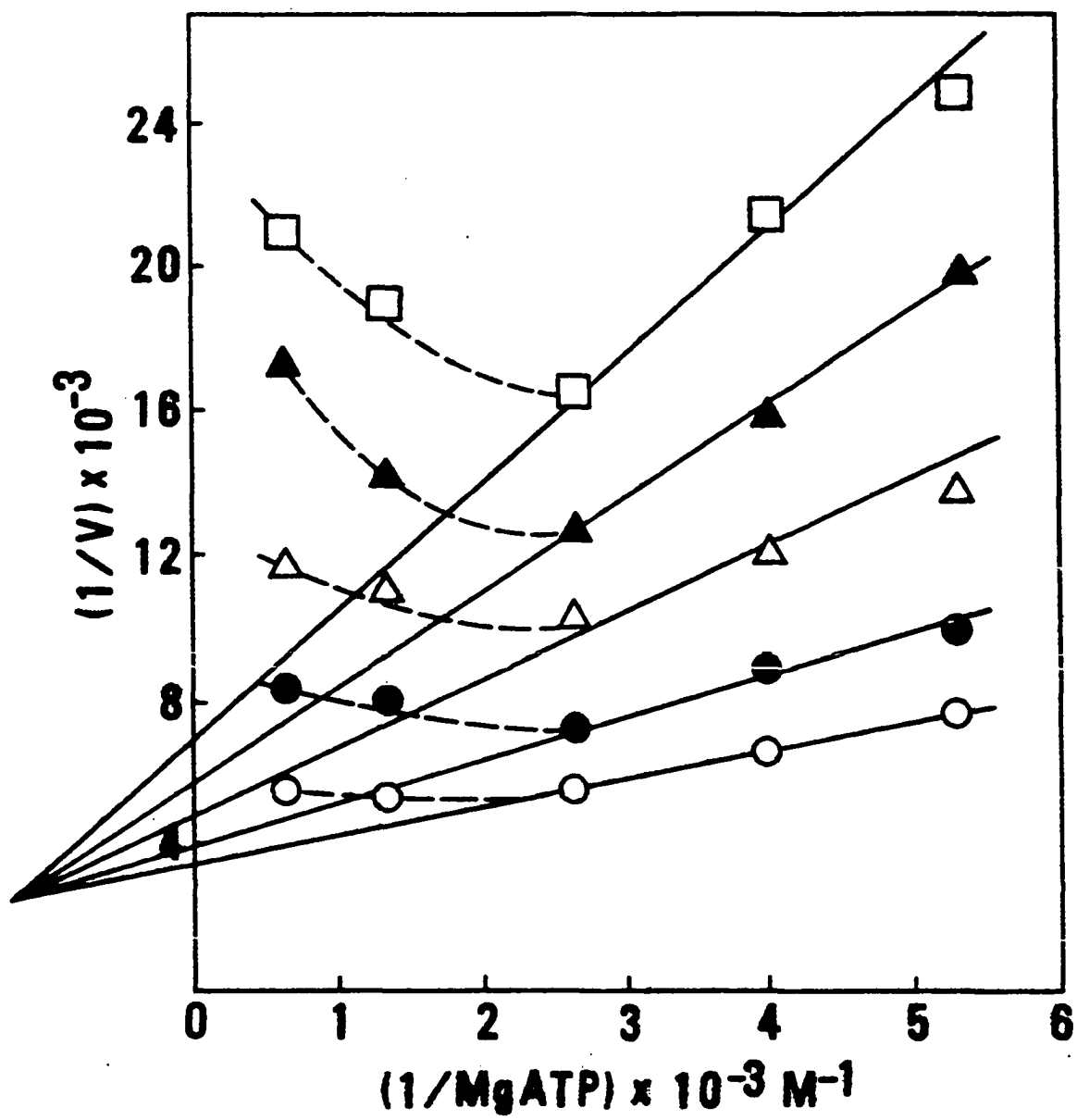


Figure 14. Double reciprocal plot of initial velocity versus MgATP concentration at constant phosphorylase b concentrations. The reciprocal of the initial velocities at several but constant levels of phosphorylase b were plotted as a function of the reciprocal of MgATP concentration. The incubation mixture contained 0.38  $\mu\text{g/ml}$  of activated phosphorylase kinase, 50 mM Tris-HCl pH 7.6, 1 mM DTE, and 2 mM free  $\text{Mg}^{2+}$  in addition to  $\text{Mg}^{32}\text{P-ATP}$  and phosphorylase b. Phosphorylase b concentrations were:  
 $\square$  8.7  $\mu\text{M}$ ,  $\Delta$  11.6  $\mu\text{M}$ ,  $\triangle$  17.5  $\mu\text{M}$ ,  $\bullet$   $\mu\text{M}$ ,  
 $\circ$  70  $\mu\text{M}$



at the same time affecting the  $\text{ATP}^{4-}$  level. When higher free  $\text{Mg}^{2+}$  levels were used, the upward curvature was no longer noticed in the peptide experiments. The  $K_m$  and  $V_m$  values of peptide and phosphorylase b for phosphorylase do not change significantly when varying the  $\text{Mg}^{2+}$  concentration from 2 - 10 mM (not shown).

The secondary plots constructed from the primary plots with phosphorylase b as substrate are shown in Figures 15 and 16.  $K_b$  of MgATP for phosphorylase kinase was calculated from the slope (closed circles) of the intercept plot. The  $K_a$  of phosphorylase b could not be calculated from this plot, as the plot is curvilinear (open circles), but could be calculated from the slope plot shown in Figure 16. The maximal velocity,  $V_1$ , was calculated from the common intersection point of the intercept plot shown on Figure 15. The results of the calculations from the initial rate studies are shown in Table 1. In comparing turnover numbers of phosphorylase b as substrate it is very interesting to note that the kinase reaction occurs at half the efficiency with tetradecapeptide. This is all the more interesting when considering the large difference in size of the two substrates. The Michaelis constant for MgATP is the same with either substrate, while the Michaelis constants for the phosphorylatable substrates differ by a factor of 64. The formulae of the rate equations used for calculation of the results in Table 1 are shown in



Figure 15. Intercept plot. Intercepts of the family of curves with the  $1/v$  axis of Figures 13 and 14 are plotted versus the reciprocal of MgATP and phosphorylase b concentrations, respectively. ● MgATP, ○ phosphorylase b

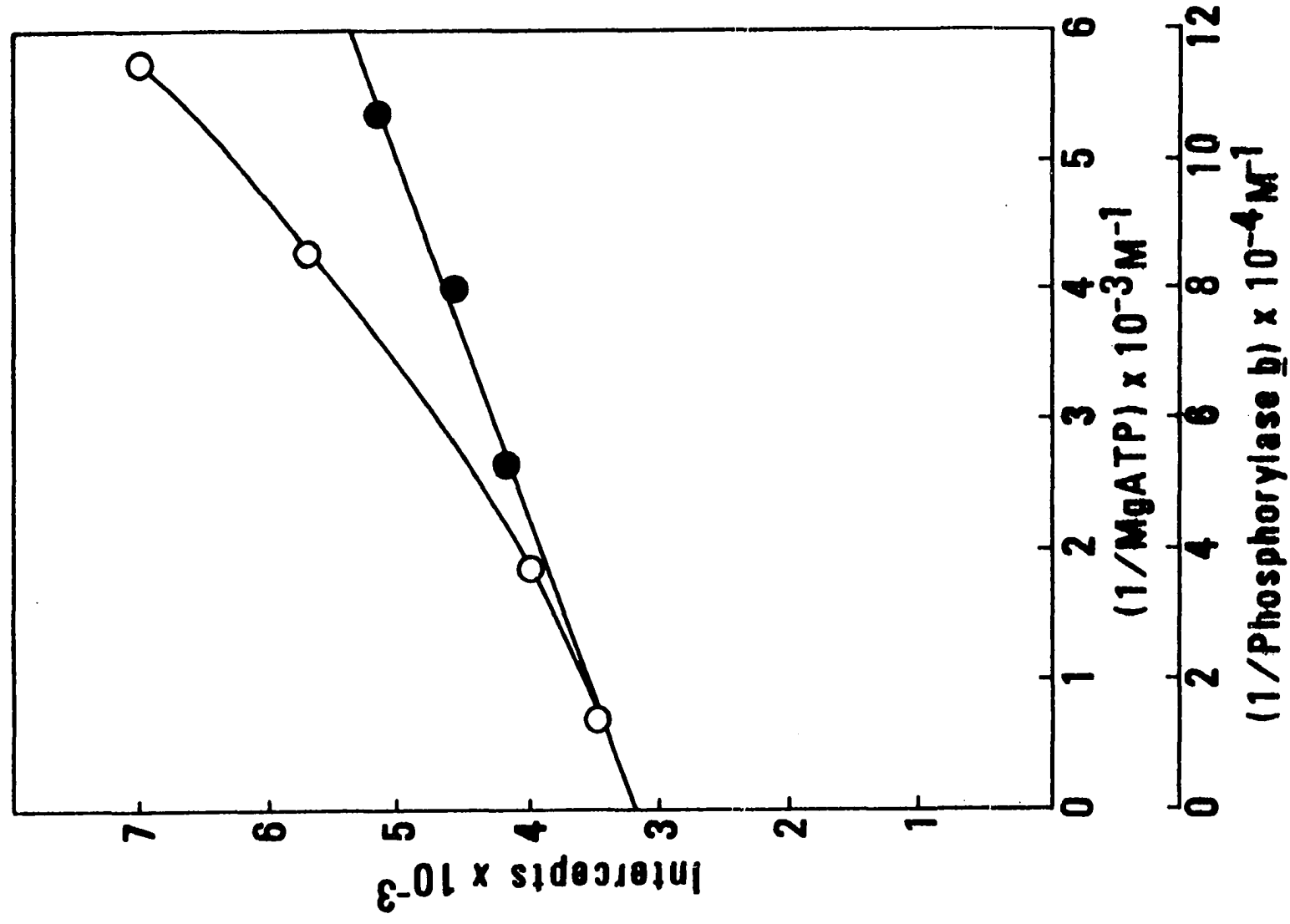


Figure 16. Slope plot. Slopes of the family of curves of Figures 13 and 14 are plotted versus the reciprocal of MgATP and phosphorylase b concentrations. ● MgATP, ○ phosphorylase b

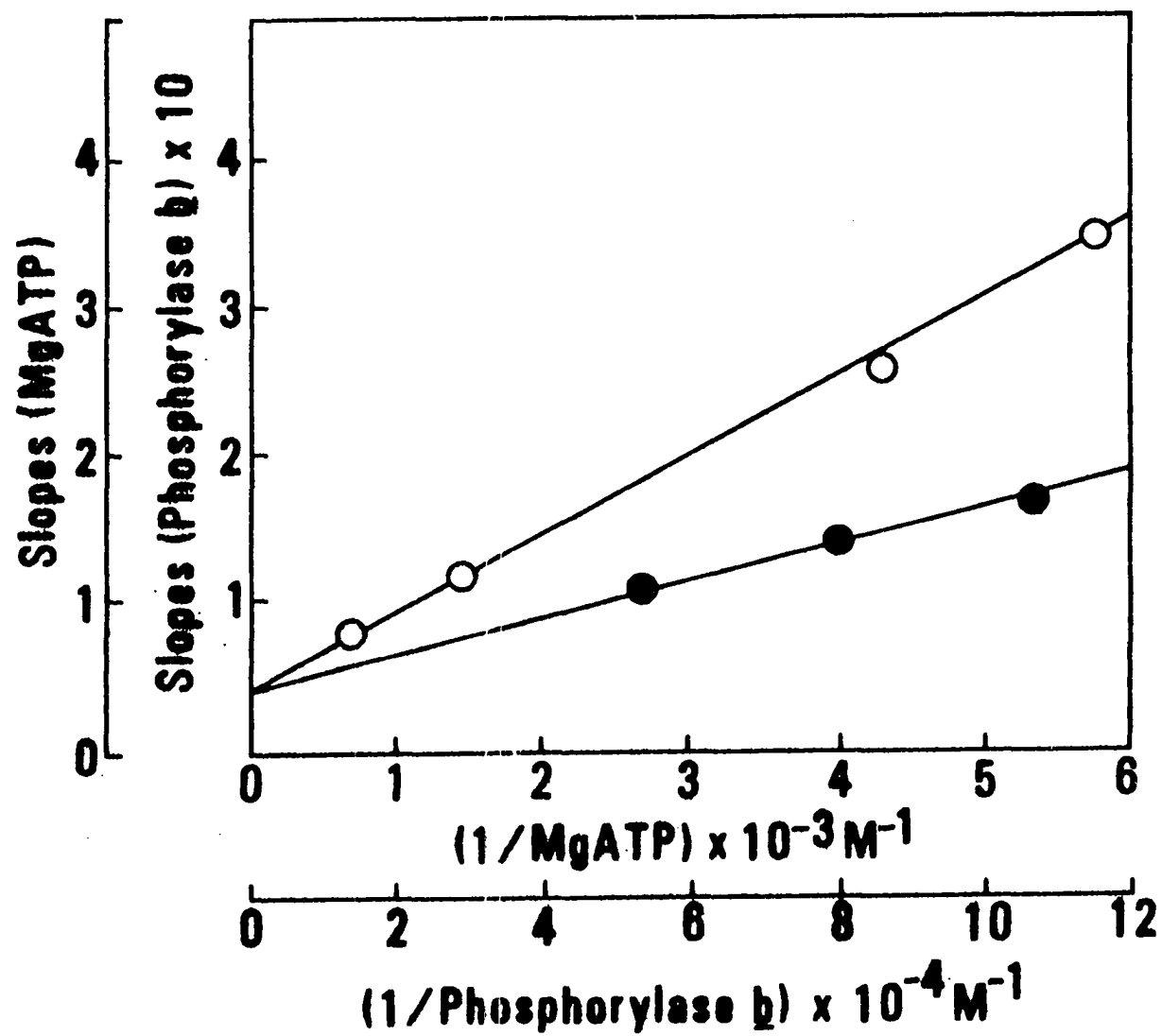


Table 1. Kinetic parameters of activated phosphorylase kinase

Assay system	$K_a^a$ (mM)	$K_b^b$ (mM)	$V_1$ ( $\mu$ mole/min/mg)
Tetradecapeptide	0.87	0.14	7.14
Phosphorylase <u>b</u>	0.0125	0.12	15.60

<sup>a</sup>Michaelis constant of peptide or phosphorylase b at infinite  $MgATP^{2-}$  concentration.

<sup>b</sup>Michaelis constant of  $MgATP^{2-}$  at infinite peptide or phosphorylase b at concentration.

Appendix B. Thus from initial rate studies using both tetradecapeptide and phosphorylase b as substrates, it can be concluded that both reactions catalyzed by phosphorylase kinase are consistent with the Sequential model (i.e., products are released from the enzyme after both substrates are bound). This is described by the following rate equation:

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1(A)} + \frac{K_b}{V_1(B)} + \frac{K_{ia} K_b}{V_1(A)(B)} \quad (\text{Appendix B}).$$

#### Choice of competitive inhibitors of substrates

In order to determine the type of kinetic mechanism of phosphorylase kinase, i.e., whether or not there is a required order for addition of substrates (Ordered or Random addition), substrate analogues can be used as competitive inhibitors to distinguish between these two mechanisms (Fromm and Zewe, 1962). To this end, seven synthetic peptides which bind reasonably well to kinase, as evidenced by their  $K_m$  value, but which are poorly phosphorylated (Tessmer, 1975) were screened for their ability to cause inhibition of tetradecapeptide and phosphorylase b phosphorylation. The results are shown in Table 2. There is only one peptide out of the seven screened which caused activation of the phosphorylation reaction towards both substrates used. Two peptides, S-4 and S-6, activated only the phosphorylation of phosphorylase b.

The remaining four peptides caused inhibition of the phosphorylation reaction of both the synthetic and the natural substrate. Coincidentally, three of these four peptides have an arginine residue at the amino- as well as at the carboxy-terminal of the peptide. On the basis of the data in Table 2, peptide S-21 was chosen since its inhibitory effects were similar when phosphorylase b or tetradecapaptide was used as substrate.

Several substrate analogues as potential competitive inhibitors of ATP were screened also. Included were  $\text{ATP}^{4-}$  which is a potent inhibitor of phosphorylase kinase (Krebs et al., 1964), AMP, ITP, and AMPPCP.  $\text{ATP}^{4-}$ , although an excellent known inhibitor of the kinase reaction could not be used here because of the requirement of a relatively high free  $\text{Mg}^{2+}$  level (millimolar range) for kinase activity (Clerch and Huijing 1972; Krebs et al., 1964).

MgAMP did not give linear inhibition kinetics (not shown).  $\text{MgITP}^{2-}$  served neither as substrate, nor as a competitive inhibitor of  $\text{MgATP}^{2-}$  (not shown). The analogue of ATP, AMPPCP, which cannot serve as substrate for the kinase reaction, because kinase cannot transfer the  $\gamma$  phosphoryl group. This analogue does behave as a competitive inhibitor of  $\text{MgATP}^{2-}$  in the phosphorylase kinase reaction as observed in preliminary experiments (not shown).

Double reciprocal plots of initial velocity versus

Table 2. Effect of potential peptide inhibitors on the conversion of tetradecapeptide and phosphorylase b

ser-asp-gln-glu-lys-arg-lys-gln-ile-ser-val-arg-gly-leu													
1 2 3 4 5 6 7 8 9 10 11 12 13 14													
Peptide Inhibitor	Sequence <sup>a</sup>			Concentration in assay (mM)			K <sub>m</sub> <sup>b</sup> (mM)		Tetradeca/peptide <sup>c</sup> conversion % A/A <sub>0</sub>			Phosphorylase <sup>b</sup> <sub>c</sub> conversion % A/A <sub>0</sub>	
S-6	5-12			1.1			0.2		50			116	
S-1	6-12			1.7			0.4		61			71	
S-5	6-12 (6-lys)			3.2			0.8		45			68	
S-2	6-12 (8-glu)			2.8			0.8		42			56	
S-21	6-12 (10-thr)			3.8			--		29			38	
S-4	7-12			1.3			--		67			121	
S-10	8-14			2.1			0.9		126			177	

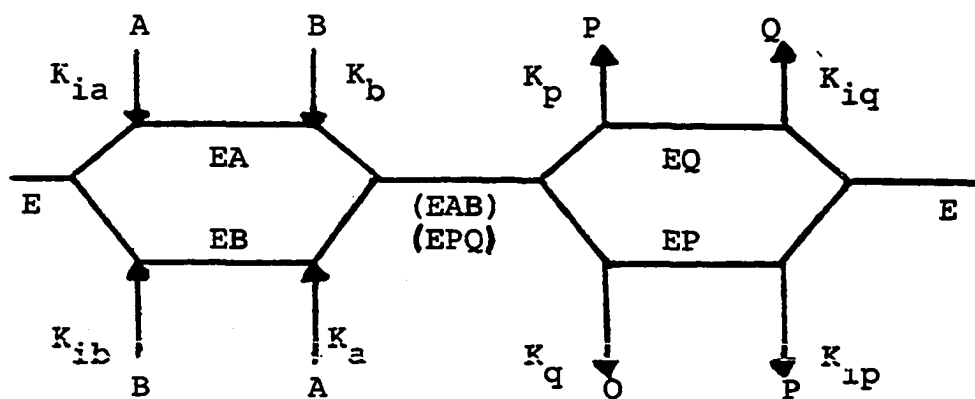
<sup>a</sup>Sequence of tetradecapeptide, number and residue in parentheses indicates point of substitution and amino acid substituted.

<sup>b</sup>Taken from Tessmer (1975).

<sup>c</sup>Relative activities of activated phosphorylase kinase with and without inhibitor.



substrate concentration at various constant levels of substrate analogue are shown on Figures 17-20 using the peptide analogue, and on Figures 21-24 using the ATP analogue. From these results it is immediately evident that the substrate analogues show competitive (with substrate) and noncompetitive (with other substrate) behaviour. The kinetic mechanism, therefore, is of the Random type and can be described by the following scheme (Fromm, 1975):



The nonlinear, parabolic concave-up curve on the secondary plot with peptide inhibitor (Figure 19) could indicate that the inhibitor adds more than once to the enzyme, i.e., there is more than one enzyme-inhibitor form:  $E + I \rightarrow EI$ ,  $EI + I \rightarrow EI_2$ , etc. (Fromm, 1975). Support for the idea, that there may be more than one site for S-21 peptide on kinase comes from the work of Carlson and Graves (1976). From their experiments showing a lack of inhibition of

Figure 17. Kinetics of inhibition by threonine-heptapeptide relative to the tetradecapeptide. The reciprocal of initial velocity is plotted versus the reciprocal of tetradecapeptide concentration at various but constant levels of thr-heptapeptide. The incubation mixtures contained 50 mM Tris·HCl pH 7.6, 1 mM DTE, 0.15 mM  $^{32}\text{P}$ -ATP ( $K_m$  concentration), 10 mM free  $\text{Mg}^{2+}$ , and 3.7  $\mu\text{g/ml}$  of activated phosphorylase kinase. Thr-heptapeptide concentrations were:  $\blacktriangle$  2.85 mM,  $\triangle$  1.90 mM,  $\bullet$  0.95 mM,  $\circ$  none

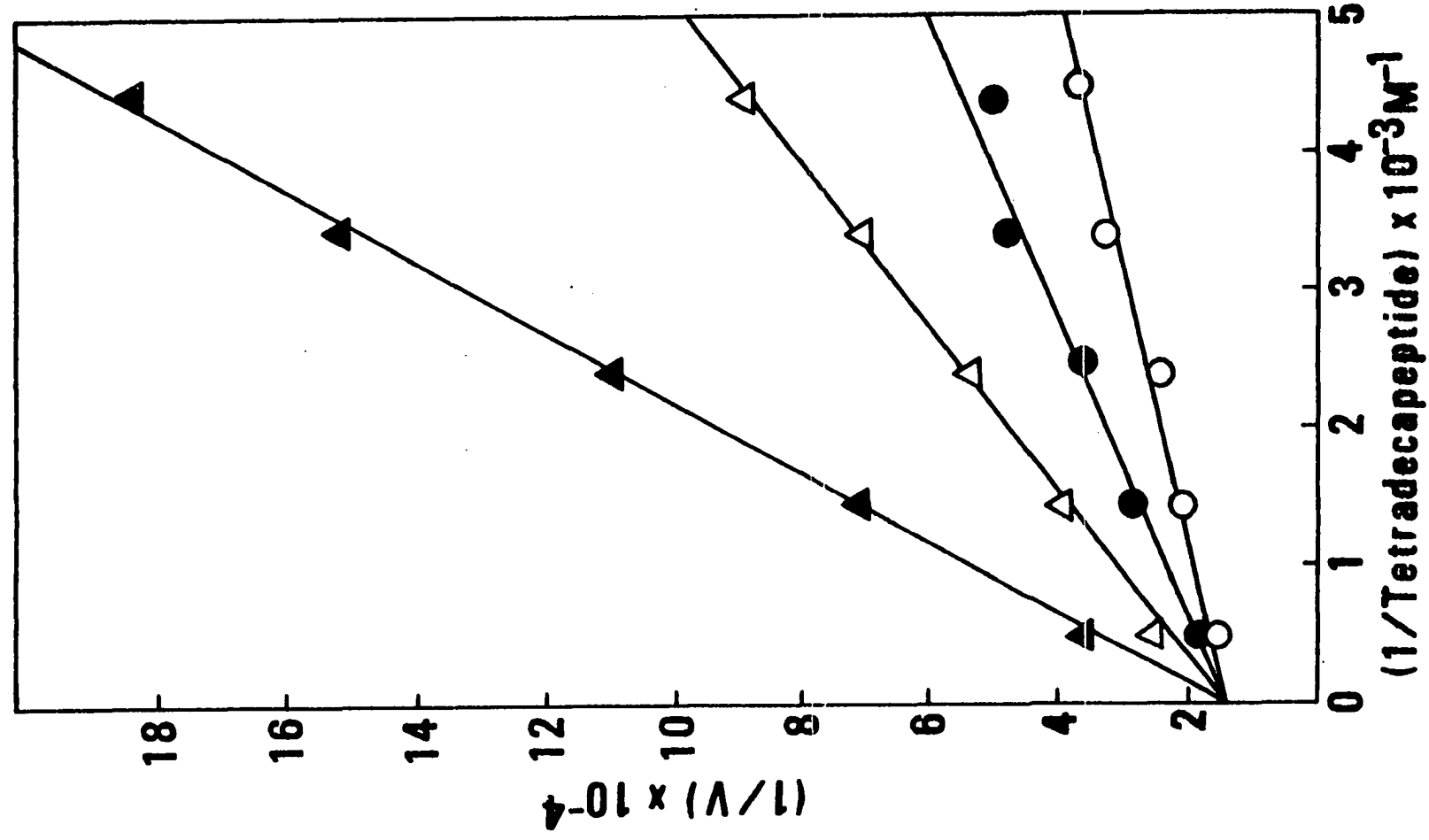


Figure 18. Kinetics of inhibition by threonine-heptapeptide relative to MgATP. The reciprocal of initial velocity versus the reciprocal of MgATP concentration at various but constant levels of thre-heptapeptide. The incubation mixtures contained 50 mM Tris·HCl pH 7.6, 1 mM DTE, 0.8 mM tetradecapeptide ( $K_m$  concentration), 10 mM free  $Mg^{2+}$ . Thr-heptapeptide concentrations were: ▲ 2.85 mM, Δ 1.90 mM, ● 0.95 mM, ○ none

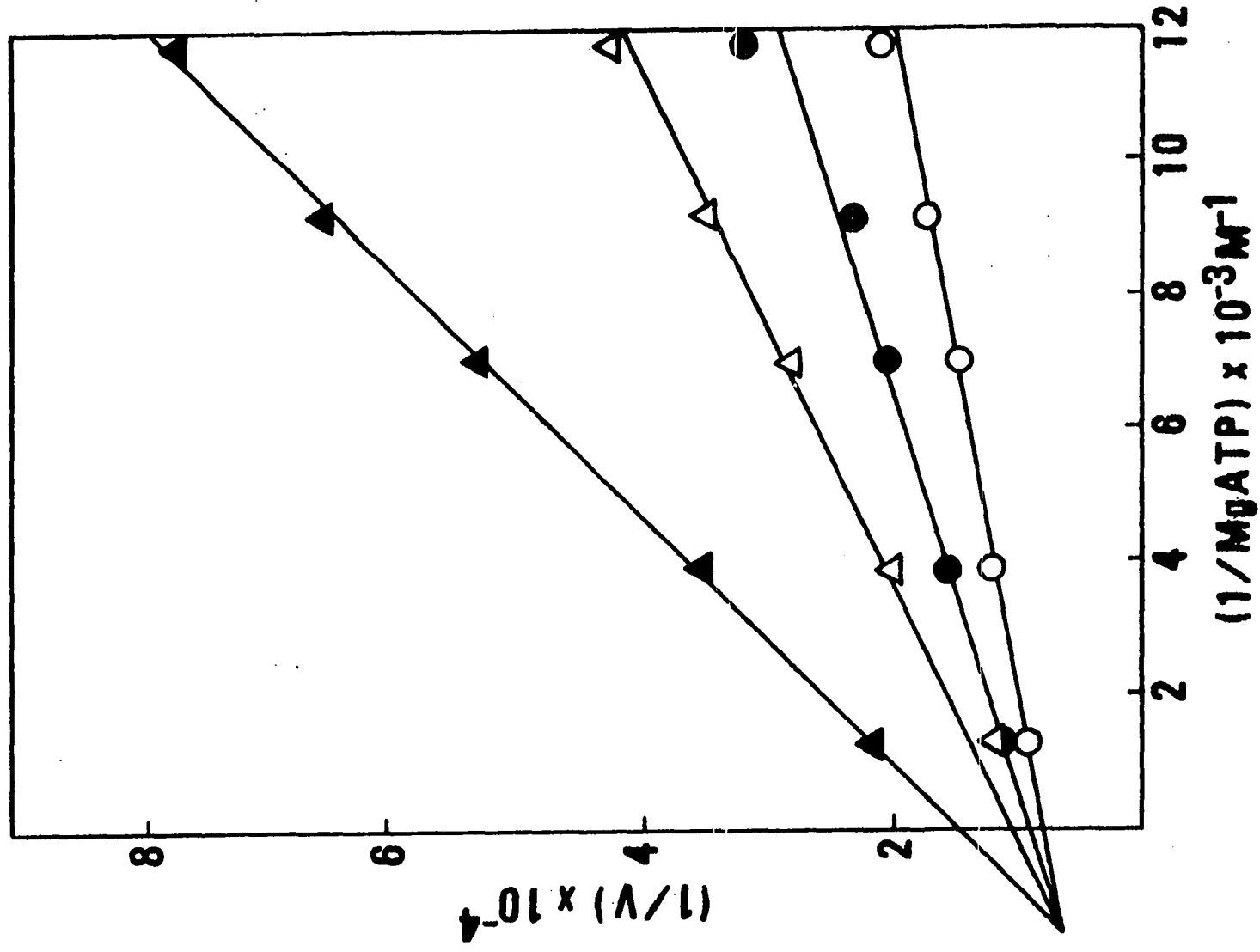


Figure 19. Slope plot derived from primary inhibition plot. Slopes are calculated from the family of curves of Figure 17 and are plotted as a function of Thr-peptide concentration

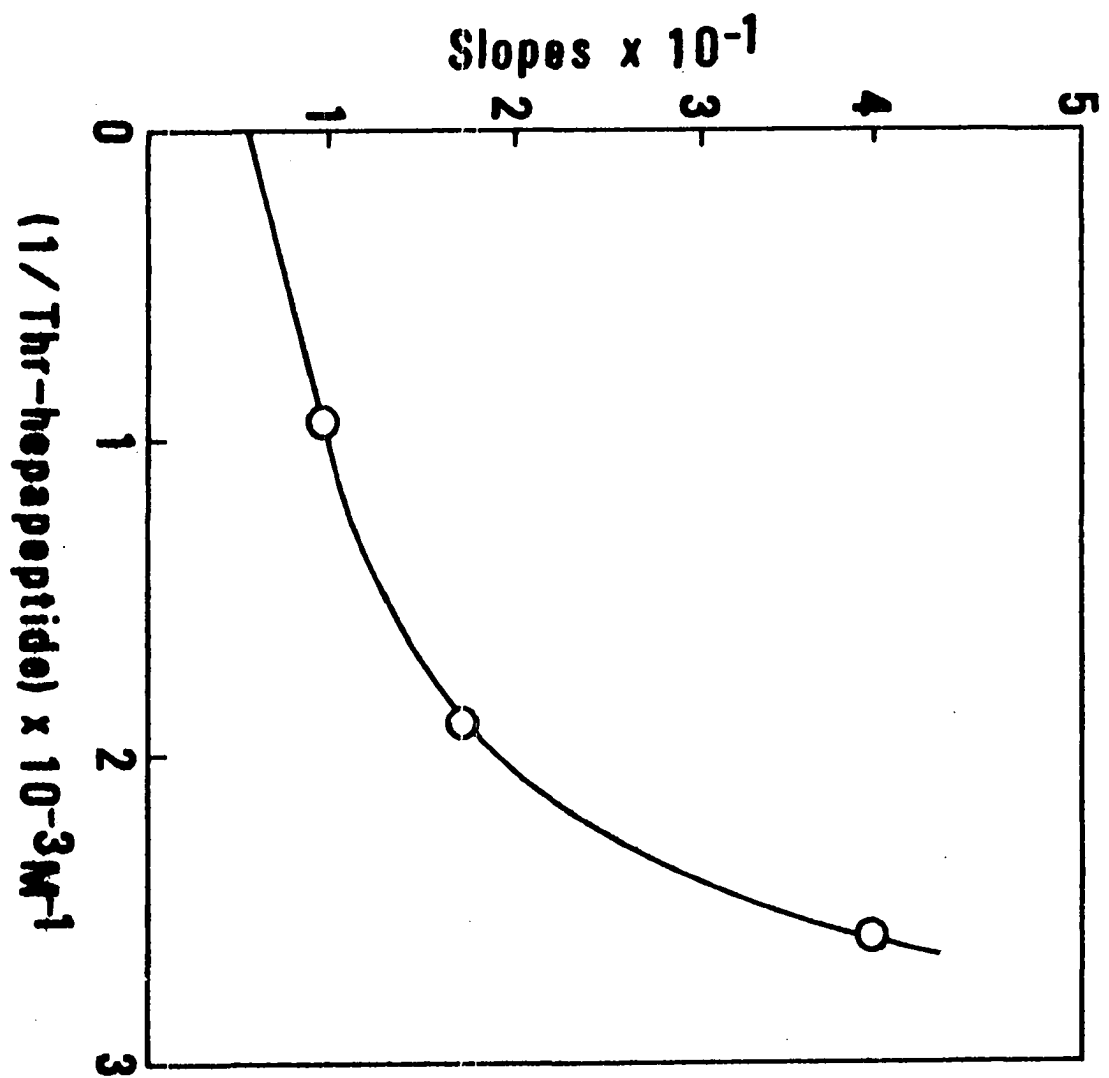


Figure 20. Intercept and slope plot derived from the primary inhibition plot. Intercepts and slopes are taken from the  $1/v$  axis and family of curves of Figure 18, respectively, and are plotted as a function of thr-heptapeptide concentration.  
● intercepts, ○ slopes



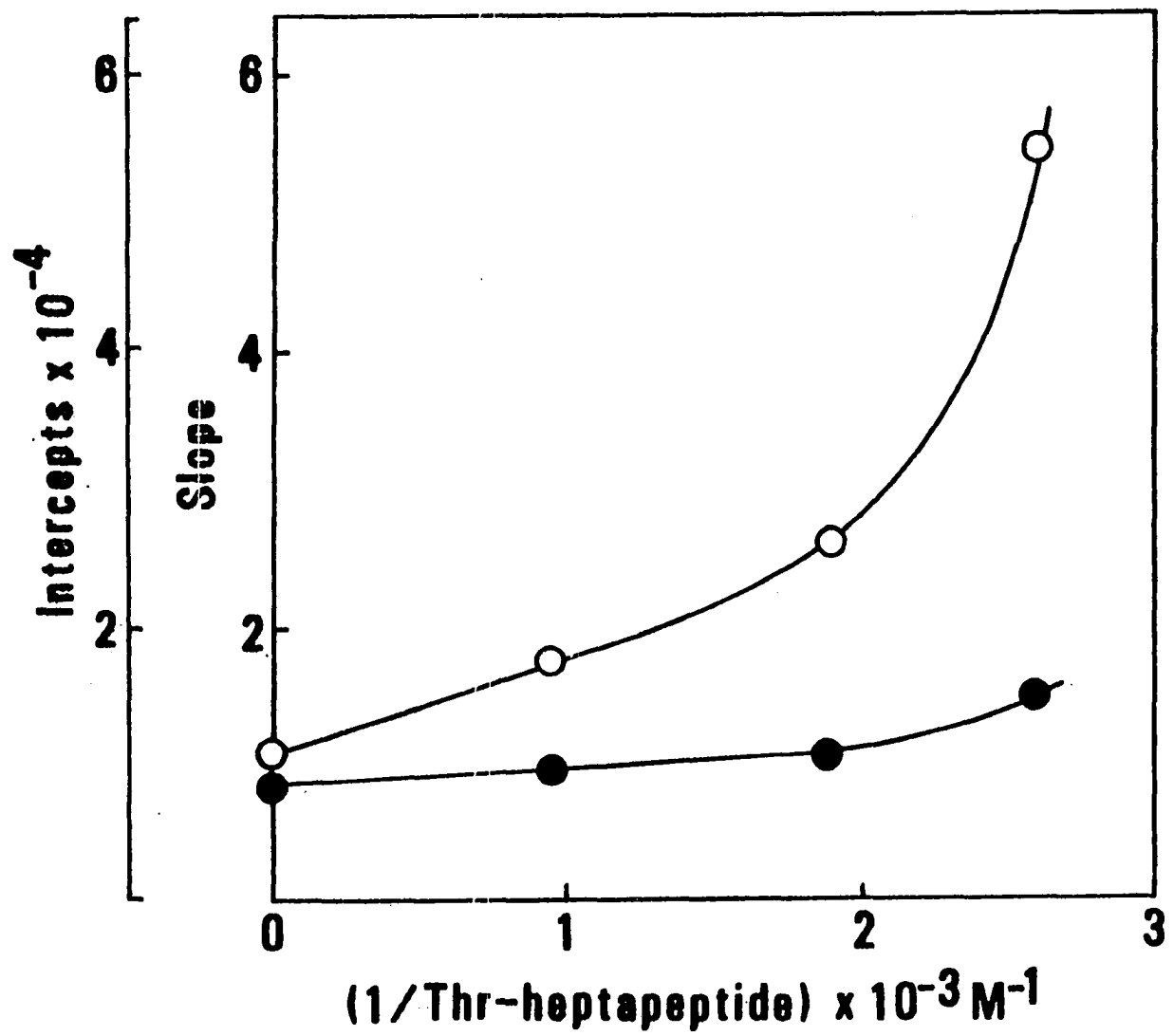


Figure 21. Kinetics of AMPPCP inhibition relative to MgATP. The reciprocal of initial velocity was plotted versus the reciprocal of MgATP concentrations at various but constant levels of AMPPCP. The incubation mixtures contained 6  $\mu\text{g/ml}$  of activated phosphorylase kinase, 50 mM Tris·HCl pH 7.6, 1 mM DTE, 10 mM free  $\text{Mg}^{2+}$ , 0.8 mM tetradecapeptide ( $K_m$  concentration) in addition to  $\text{Mg}^{32}\text{P-ATP}$  and MgAMPPCP. MgAMPPCP concentrations in mmol/l were as indicated on the figure

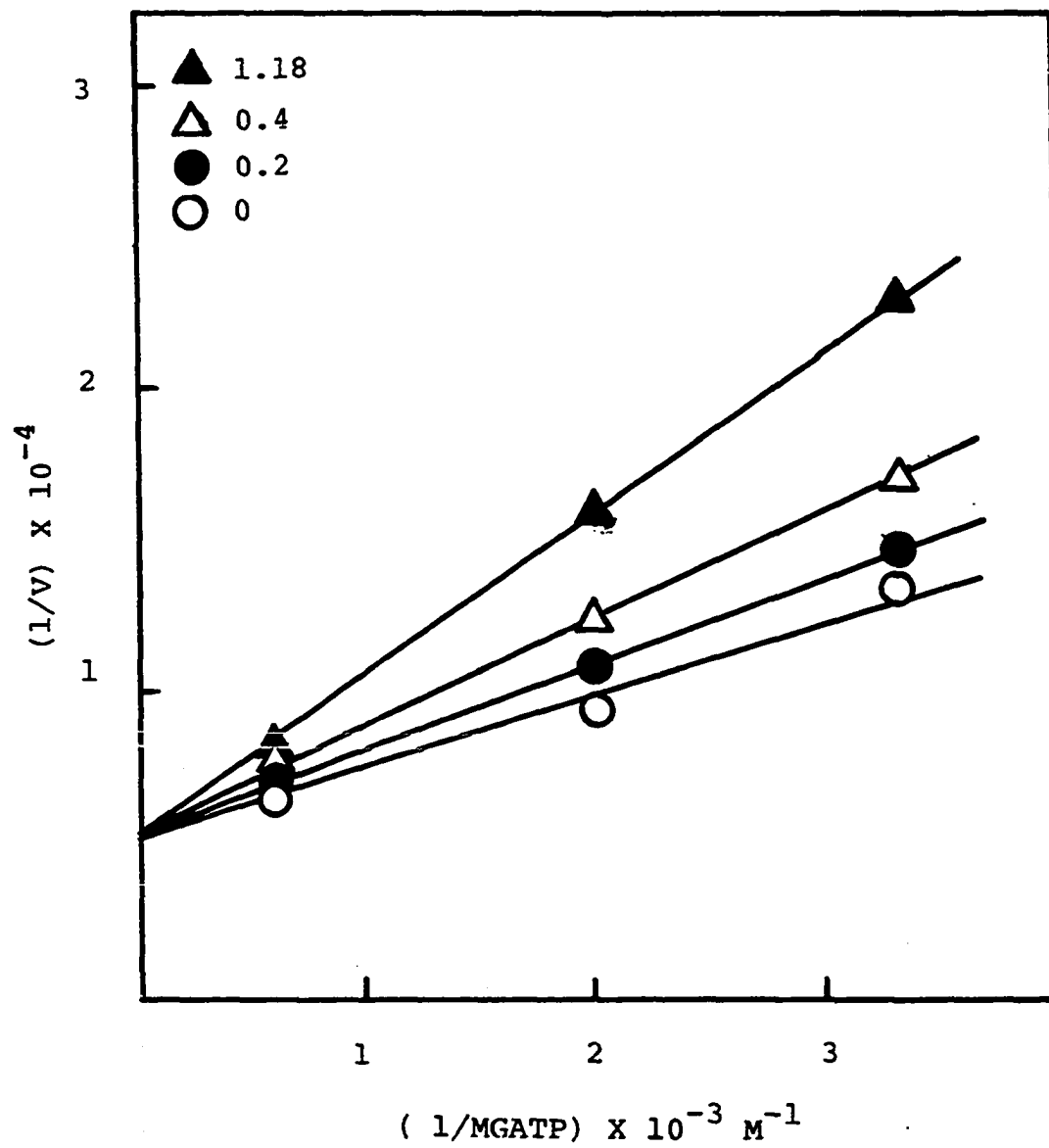


Figure 22. Kinetics of AMPPCP inhibition relative to tetradecapeptide. The reciprocal of initial velocity was plotted versus the reciprocal of tetradecapaptide concentrations at various but constant levels of AMPPCP. The incubation mixtures contained 10.4  $\mu\text{g/ml}$  of activated phosphorylase kinase, 50 mM Tris HCl pH 7.6, 1 mM DTE, 10 mM free  $\text{MgCl}_2$ , 0.15 mM  $\text{Mg}^{32}\text{P-ATP}$  ( $K_m$  concentration) in addition to tetradecapeptide and  $\text{MgAMPPCP}$ .  $\text{MgAMPPCP}$  concentrations in mmole/l were as indicated on the figure

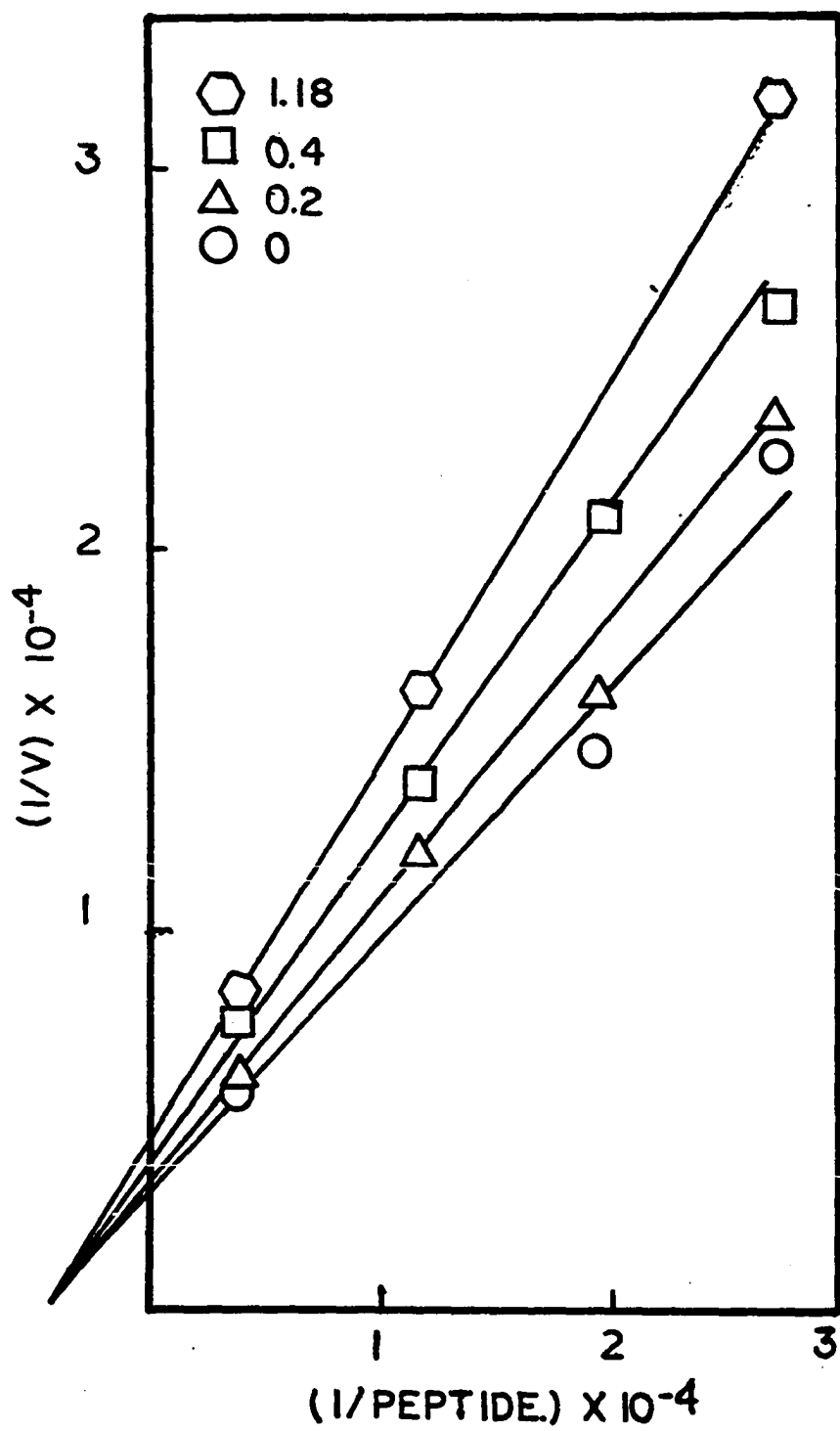


Figure 23. Slope plot derived from primary inhibition plot. Slopes are calculated from the family of curves of Figure 21, and are plotted as a function of AMPPCP concentration

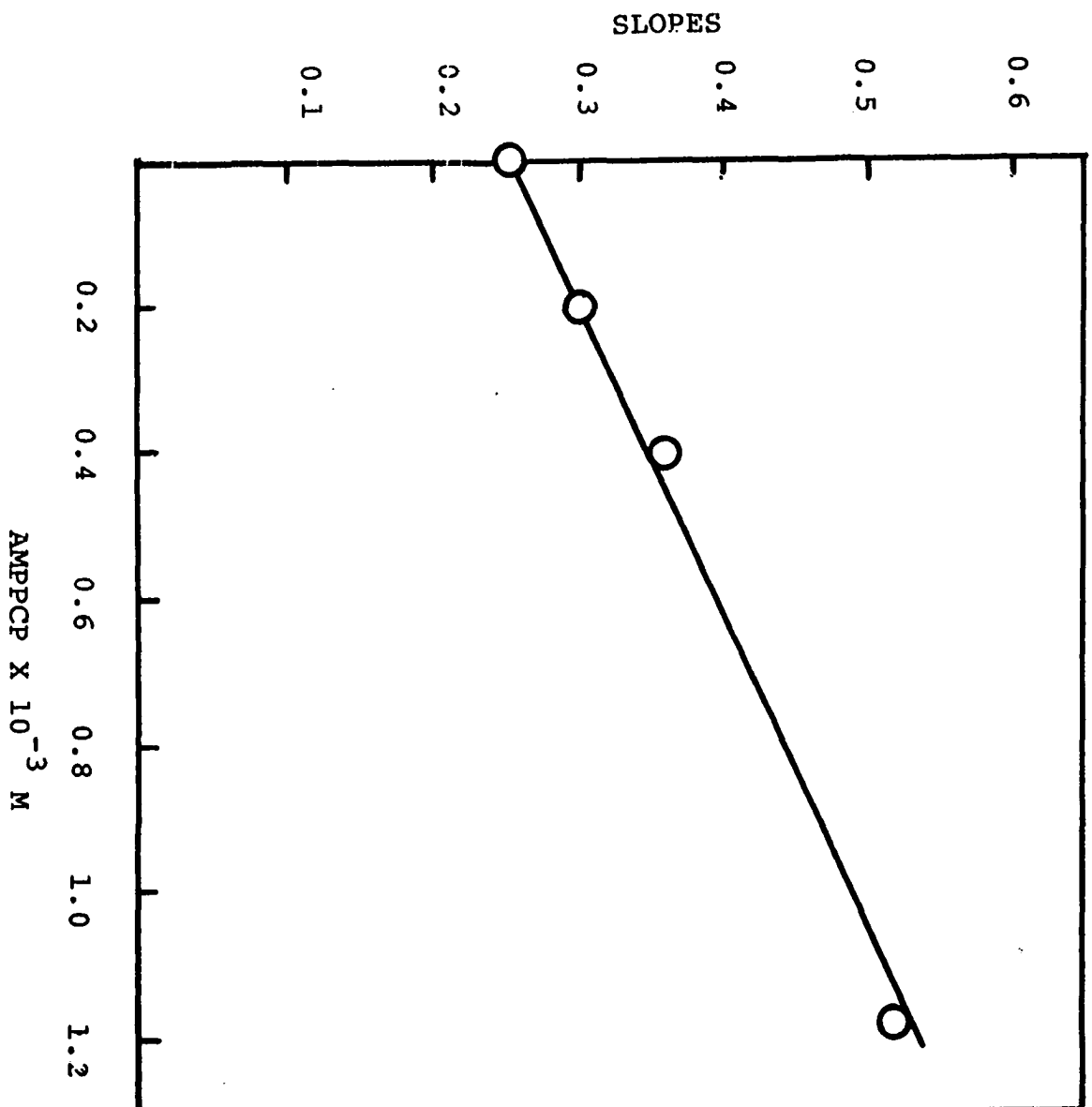
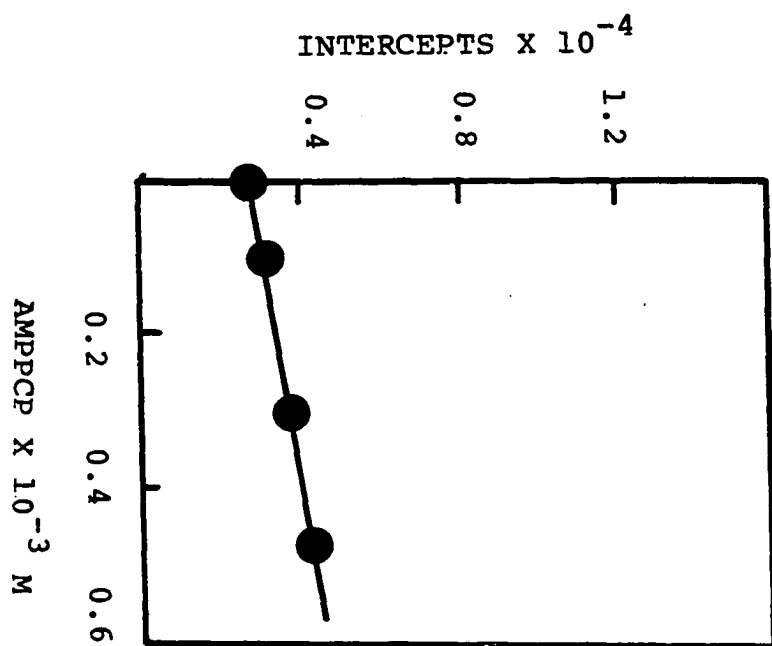
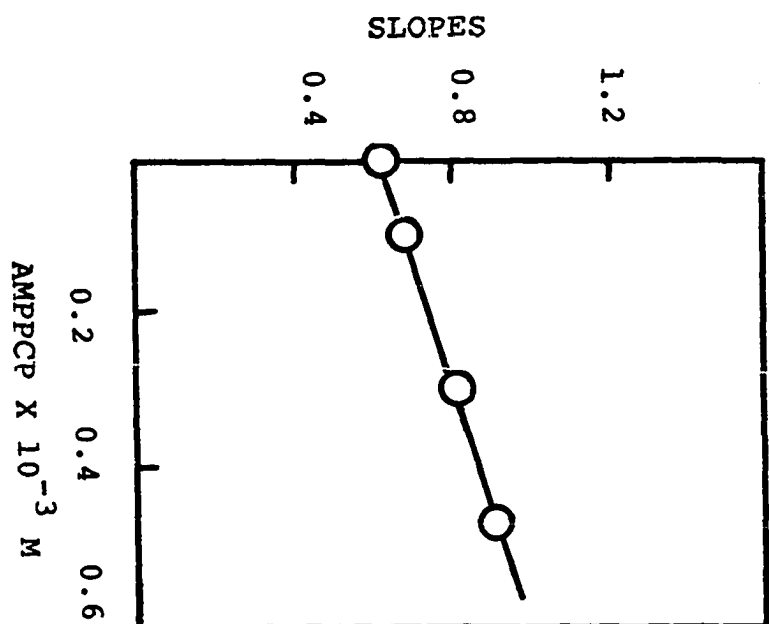


Figure 24. Slope and intercept plot derived from primary inhibition plot. Slopes and intercepts are calculated from Figure 22 and plotted as a function of AMPPCP concentration





autophosphorylation by peptide S-21 under saturating conditions of tetradecapeptide, suggested there could be allosteric site(s) for peptides on phosphorylase kinase.

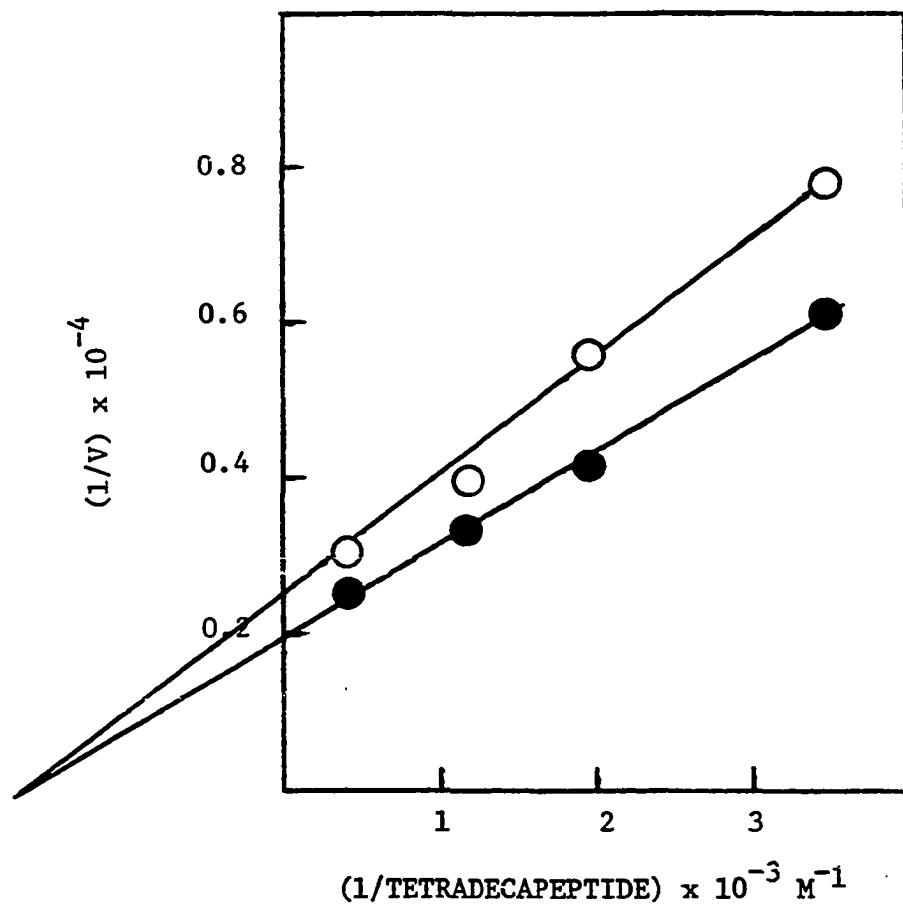
### Effect of glycogen

It had been reported in the literature (Krebs et al., 1964) that the presence of glycogen decreases the  $K_m$  of phosphorylase b either through conformational changes by the glycogen-phosphorylase interaction or by the glycogen-phosphorylase kinase interaction, or both. If it is assumed that there is minimal interaction between glycogen-peptide then it follows that changes in the kinetic parameters would then be due to glycogen-enzyme interaction. The kinetic experiments performed are shown in Figure 25. It is apparent that the effect of glycogen is only on the  $V_m$ .

### Reversal of the phosphorylase kinase reaction

Attempts to reverse the phosphorylase kinase reaction in the presence of  $^{32}\text{P}$ -tetradecapeptide (1 mM), ADP(1 mM) and high  $\text{Mg}^{2+}$  (20 mM) have been unsuccessful in our hands. No  $^{32}\text{P}$ -ATP formation was detected by radioautography after high voltage paper electrophoresis was performed on the reaction mixture. Lowering the pH to 6.5 also did not cause formation of detectable  $^{32}\text{P}$ -ATP. It should be pointed out that the high  $\text{Mg}^{2+}$  levels used could cause inactivation of phosphorylase kinase (Krebs et al., 1964). Attempts to reverse the

Figure 25. Effect of glycogen on the apparent  $K_m$  and  $V_m$  of tetradecapaptide. The incubation<sup>m</sup> mixtures contained 50 mM Tris·HCl pH 7.6, 1 mM DTE, 1.5 mM  $Mg^{32}P$ -ATP, 10 mM free  $Mg^{2+}$ , and 8  $\mu$ g/ml of activated phosphorylase kinase in addition to tetradecapeptide.  
● no glycogen, ○ 0.4% glycogen



phosphorylase kinase reaction with phosphorylase b as substrate were also not successful (Krebs et al., 1964).

#### Substrate specificity of phosphorylase kinase

The efficiency of the phosphorylase kinase reaction with tetradecapaptide was surprisingly good compared to its natural substrate, phosphorylase b, as shown in Table 1. An interesting question can now be posed: how small can the peptide substrate be; which essential features must the peptide possess in order to bind well to kinase and for catalysis to take place? To this end, several peptides were used in kinetic studies to evaluate the  $K_m$  and relative  $V_m$  values. The peptides chosen had specific substitutions or modifications,

- i length of peptide
- ii hexapeptides with a C-terminal arginine, C-terminal arginine-amide with and without an acetylated N-terminal lysine
- iii peptides substituted at the 12-arg position by alanine or lysine.

The kinetic parameters of the phosphorylase kinase reaction were compared to tetradecapaptide, which was used as the reference peptide in each of the three studies referred to above. Since some of the peptides studied do not bind to phosphocellulose paper, the extent of  $^{32}\text{P}$ -peptide formation was measured using the column method. Thus, the results

shown in Tables 3 and 4 were obtained by measuring  $^{32}\text{P}$ -peptide formation using the column method and results shown in Table 5 were obtained by measuring  $^{32}\text{P}$ -peptide bound to phosphocellulose paper. Some of the phosphorylase kinase preparations used were stored somewhat longer than others before use. Because of this, some differences were noted in the  $K_m$  values. The relative differences in  $K_m$  values and  $V_m$  values of peptides with a particular phosphorylase kinase preparation, however, were the same. The data in Table 3 indicate that the smallest peptide, a hexapeptide (Ile-HP), binds surprisingly well to the kinase compared to tetradecapeptide (S-30), even though the hexapeptide is largely hydrophobic in character. The catalytic efficiency of the kinase reaction with this peptide, however, is very poor (Table 3). Since the phosphorylase kinase reaction showed nonlinear kinetics with this peptide (not shown) it is possible that indeed there could exist an allosteric site(s) on kinase for this small peptide. In addition, our results with the threonine peptide (S-21) would agree with this observation, as this peptide was found to be a nonlinear competitive inhibitor of tetradecapeptide in the phosphorylase kinase reaction (Figures 17 and 19). As the peptide length is increased, the relative  $V_m$  is increased also, but the  $K_m$  values do not change significantly compared to tetradecapeptide (S-30). Evidently, amino acid residues 1-8 of the sequence

Table 3. Effect of length of peptide on the  $K_m$  and  $V_m$ <sup>a</sup>

Ser-asp-gln-glu-lys-arg-lys-gln-ile-ser-val-arg-gly-leu													
	1	2	3	4	5	6	7	8	9	10	11	12	13 14
Peptide	Sequence						Substitution		$K_m$ (mM)		% Relative $V_m$		
S-30	1-14						none		2.9		100		
Ile-HP <sup>b</sup>	9-14						none		1.5		2.5		
S-10	8-14						none		3.3		12		
S-11	7-14						none		2.5		29		

<sup>a</sup> Assays were performed under the following conditions. Incubation mixtures at 30°C contained, 1.5 mM  $^{32}\text{P}$ -ATP, 10 mM free  $\text{Mg}^{2+}$ , 50 mM Tris·HCl pH 7.6, 1 mM DTE, 6.7  $\mu\text{g/ml}$  activated phosphorylase kinase and peptides at various concentrations. Initial velocities were calculated from 15 min time points for the poor substrates and from 4 min time points for the good substrates. Linearity of the rate of product formation had previously been established.

<sup>b</sup> Phosphorylase kinase showed nonlinear kinetics with this peptide. The  $V_m$  was estimated from the nonlinear double reciprocal plot. The  $S_{0.5}$  was obtained after evaluating the Hill coefficient from the velocity form of the Hill equation and replotting the initial velocity data as a function of the concentration of peptide raised to the power of the Hill coefficient (Fromm, 1975) in a double reciprocal plot.

Table 4.  $K_m$  and relative  $V_m$  of various C-terminal arginine containing hexapeptides<sup>a</sup>

Ser-asp-gln-glu-lys-arg-lys-gln-ile-ser-val-arg-gly-leu													
1	2	3	4	5	6	7	8	9	10	11	12	13	14
Peptide	Sequence			Substitution			K <sub>m</sub> (mM)		% Relative V <sub>m</sub>				
S-30	1-14			none			1.4		100				
S-4	7-12			none			1.1		2-3.6				
HP-amide	7-12			12-argNH <sub>2</sub>			1.3		7				
Ac-HP-amide	7-12			7-Aclys.			2.5		15-22				
				12-argNH <sub>2</sub>									

<sup>a</sup>The conditions of the assay were the same as described in footnote "a" of Table 3.



Table 5. Effect of substitution at the 12-arg position on  $K_m$  and relative  $V_m$ <sup>a</sup>

Ser-asp-gln-glu-lys-arg-lys-gln-ile-ser-val-arg-gly-leu													
1 2 3 4 5 6 7 8 9 10 11 12 13 14													
Peptide	Sequence					Substitution				$K_m$ (mM)	% Relative $V_m$		
S-30	1-14					none				0.8	100		
S-24	5-14					12-ala				2.4	10		
Lys-pep	6-13					6-lys, 12-lys				2.0	2.5		

<sup>a</sup>Conditions of the assay were the same as described in footnote "a" of Table 3.

of tetradecapeptide (Table 3) contribute very little to the  $K_m$  of peptide for phosphorylase kinase, but these residues might contribute to a more favorable conformation of the peptide. In Table 4 are shown the results obtained with the C-terminal arginine hexapeptides. These peptides had similar affinity for activated phosphorylase kinase as tetradecapeptide, except for one peptide, the acetylated hexapeptide containing a C-terminal arginine amide. The relative  $V_m$  increases as the negative charge on the C-terminal side and the positive charge on the N-terminal side are removed by modification reactions. The results observed with the unmodified hexapeptide (S-4) with respect to the  $V_m$  was similar to that observed by Tessmer (1975); however, we did not observe a large change in the  $K_m$  for this peptide as compared to tetradecapeptide. Tessmer (1975), using preincubated non-activated phosphorylase kinase, observed that for the C-terminal arginine peptides, sequence 1-12 and sequence 5-12, the  $K_m$  was decreased 3-fold and the  $V_m$  300 and 100-fold, respectively. They used the term "nonproductive binding" to indicate that these peptides formed a better enzyme-substrate complex than tetradecapeptide, but the peptide's orientation in the catalytic site was not completely correct. Modification of the hexapeptides increases the  $V_m$ . It is possible that these modifications now cause the peptide to bind in the correct orientation. Another possibility might

be that the modifications introduced cause the peptide to assume a more favorable conformation. It would be of interest to compare the structures of these C-terminal arginine peptides by measurement of the circular dichroism spectra.

From the above discussion of C-terminal arginine peptides it is evident that if arginine is in the proper electrostatic environment, the  $V_m$  could be raised. The question can be asked, what would happen to the  $K_m$  and  $V_m$  values when this arginine residue (12-arg) is replaced by alanine (nonconservative substitution) or by lysine (conservative substitution). The results shown in Table 5 for the alanine substituted peptide (S-24) agree with those reported by Tessmer (1975) for nonactivated preincubated phosphorylase kinase. The  $K_m$  increased 3-fold compared to tetradecapaptide (S-30) and the  $V_m$  was lowered by a factor of 10. These results are not entirely unexpected. Quite surprising, however, are the results obtained with the lysine substituted peptide. The  $V_m$  was lowered by a factor of 40 compared to tetradecapaptide. Arginine in the substrate peptide must therefore play an important role. Possible roles of arginine in the catalytic mechanism of phosphorylase kinase will be discussed in the following section.

## DISCUSSION

In the phosphorylase kinase catalyzed reaction, the macromolecular substrate, phosphorylase b is an enzyme. Regulation of the phosphorylase kinase reaction, therefore, can be achieved by effectors binding to the enzyme, to the protein substrate, or to both. Because of the multiple interactions of modifiers with enzyme and substrate, detailed kinetic studies have been difficult to do. If an alternative substrate could be used, which is not affected by modifiers, then it would be possible to perform detailed kinetic analysis. Furthermore, if such an alternative substrate were found to be a good model for the phosphorylase kinase reaction, as determined by kinetic studies, then one would be able to use this model system to study specific enzyme-substrate interactions. This could be achieved by using analogues of alternative substrates. Basic information obtained by studies of this nature, should be helpful in understanding the highly specific protein-protein interaction of phosphorylase kinase with phosphorylase b.

As with any model system one has to demonstrate that the model used is a valid one. The synthetic substrate, in other words, must mimic the natural substrate in the characteristic features of the kinase reaction. This was demonstrated with the synthetic tetradecapeptide as substrate. One of the most

important requirements is that the correct seryl residue is phosphorylated, the seryl residue located between the residues isoleucine and valine. This was shown by Tessmer and Graves (1973), by comparing fingerprints and radioautograms of a phosphopeptide isolated from a chymotryptic digest of phosphorylated phosphorylase to that of phosphorylated tetradecapeptide. Other characteristic features of the kinase reaction which are in common whether peptide or phosphorylase b are used as substrate are the pH profile (Figures 3 and 4); the lag in the rate of product formation and straightening of lag after preincubation with peptide (Figure 5) and finally, as shown in Figure 6, the gradual disappearance of the lag with increasing phosphorylase kinase concentration (Kim and Graves, 1973; Krebs et al., 1964; DeLange et al., 1968). By using the model system one avoids the many difficulties associated with studying the phosphorylase kinase reaction, where the protein substrate, phosphorylase b, has also binding sites for  $Mg^{2+}$  (Kent et al., 1958) ATP (Morgan and Parmeggiani, 1964), and glycogen (Krebs et al., 1964). The assumption that the synthetic peptide contains no binding sites for these substances is justified on the basis that ligands for binding are usually provided by amino acid side chains from different regions of the folded polypeptide chain. Only one small synthetic peptide (thr-ala-cys-gly-glu-lys-ser-pro) reportedly bound MgATP and ATP. Affinity constants

for this peptide were smaller by a factor of 500 and 50, respectively, compared to the natural substrate, myosin (Barany and Merrifield, 1972). However, because of the errors associated with the calculations of the determination of the affinity constants (Barany and Merrifield, 1972) additional approaches should be used to verify these results.

Phosphorylase kinase which had been activated through the cyclic AMP-dependent protein kinase catalyzed reaction, using the catalytic subunit, was used for the kinetic studies. This form of phosphorylase kinase had a pH 6.8 to 8.2 activity ratio of 0.3 to 0.4 as compared to 0.02 to 0.07 for nonactivated phosphorylase kinase.  $^{32}\text{P}$  incorporation into the subunits of activated kinase was used as an additional criterion for the activated form. In our hands 1.7 to 2.4 moles of phosphate were incorporated in the A subunit and 0.9 to 1.2 moles of phosphate into the B subunit. Incorporation of phosphate into the C-subunit was considered to be negligible (0.01 to 0.02 moles of phosphate per mole of C). Furthermore, the progress curves of the reaction (product formation as a function of time) with the activated form of kinase were linear, unlike those for nonactivated phosphorylase kinase. Although preincubated nonactivated phosphorylase kinase could have been used, the already complicated kinetic studies are simpler to perform when no preincubation is necessary. At this point one might ask the

question which form of phosphorylase kinase would be the more appropriate one, in a physiological context, to study. To answer this question one could argue that physiologically, the activated form of kinase has been shown to be important in the hormone stimulated glycogenolytic process. At this time no evidence has been presented that a form of phosphorylase kinase exists in vivo in muscle, which behaves like nonactivated preincubated phosphorylase kinase. Circumstantial evidence has been presented that a form of kinase modified in the presence of  $\text{Ca}^{2+}$  and MgATP (autoactivated kinase) may be important physiologically (Carlson and Graves, 1976; Wang et al., 1976). This form of phosphorylase kinase is not the same as nonactivated phosphorylase kinase, as both the activity ratio at pH 6.8 to 8.2 and the state of phosphorylation have changed. It is probably fair to assume that the kinetic studies with the activated form of kinase are relevant to nonactivated preincubated phosphorylase kinase as we have found that the specificity of the reaction has not changed.

Changes in kinetic constants of substrates as a function of the phosphorylation state of the enzyme have been observed for glycogen synthase (Roach et al., 1976). They observed that in the absence as well as in the presence of the activator glucose-6-P, the  $S_{0.5}$  (concentration of substrate giving half-maximal velocity) of UDP-glucose was dependent on the

phosphorylation state, and increased with increasing incorporation of covalently bound phosphate into the synthase molecule. The maximal velocity, however, was not affected. Similar results have been described for the kinetic constants for the two forms of phosphorylase, in the presence of the activator AMP. The maximal velocity was not very much different between the two forms, while differences were observed in the kinetic constants of the substrates (see Graves and Wang, 1972).

The initial velocity data shown in Figures 9,10,13 and 14 indicate that the mechanism of the phosphorylase kinase reaction is consistent with a Sequential model, i.e., the family of curves are not parallel, but rather they intersect. This means that the substrates remain at the active site of the enzyme before products are formed. The mechanism excluded by the initial velocity data is the Ping Pong mechanism, in which the enzyme reacts with one of the substrates to form a product that dissociates before the next substrate adds. The fact that substrate analogues for both substrates show competitive behavior relative to the like substrate and noncompetitive behavior relative to the other substrate, indicates that there occurs a random addition of substrates (Random Bi Bi mechanism). This can be explained by the fact that there are preexisting substrate binding sites on the enzyme for both substrates. In the case of the



Ordered Bi Bi mechanism there exists a substrate binding site for one of the substrates; the binding site for the second substrate becomes available after the first substrate has bound, through subtle conformational changes of the enzyme. Inhibition patterns for the Ordered mechanism would have been parallel lines instead of converging lines as shown on Figures 18 and 22. Results shown on Figure 18 with the peptide analogue indicate that an ordered path with MgATP, the obligatory first substrate to bind, can be eliminated; similarly, the results shown on Figure 22 with the ATP analogue indicate that an ordered path with peptide the obligatory first substrate to bind, can also be eliminated. This topic is extensively covered by Fromm (1975).

The kinetic parameters of the phosphorylase kinase reaction shown in Table 1 reveal an interesting aspect of the model system. Even though the Michaelis constant of the peptide ( $K_a$ ) is larger by a factor of 64 compared to the  $K_a$  of phosphorylase b, the maximal velocity ( $V_1$ ) is only about half of that when using the protein substrate. This implies that in order for the catalytic reaction to occur the peptide possesses almost all the necessary features and/or structure. This result might also be interpreted to indicate that the N-terminal portion of the polypeptide chain in native phosphorylase is readily available and that additional conformational changes of phosphorylase upon binding to phosphorylase

kinase may not be necessary. The fact that the N-terminal portion is easily accessible is consistent with the results on the ease of removal of the N-terminal peptide of phosphorylase by controlled proteolysis (Nolan et al., 1964). Although the above explanation may be tempting and oversimplified, it should be kept in mind that protein kinase has not been found to phosphorylate phosphorylase b, while the tetradecapeptide can be phosphorylated. Thus it is possible that the remainder of the phosphorylase molecule imparts a conformation to the peptide such that it is not accessible to protein kinase, or protein kinase cannot induce conformational changes so that the peptide segment becomes even more accessible. The problem of specificity will be discussed more fully in a later section.

The initial rate studies indicate that the data could be consistent with the Rapid Equilibrium Random Mechanism, i.e., the interconversion of the ternary complexes is slow compared to the other kinetic steps (see scheme, page 71) as expressed in linear double reciprocal plots (Figures 1,10,13 and 14). The values calculated for the dissociation constants of enzyme-substrate complexes ( $K_{ia}$  and  $K_{ib}$ )<sup>1</sup> depend upon the

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<sup>1</sup>When the substrate is tetradecapeptide  $K_{ia} = 1.61$  mM peptide and  $K_{ib} = 0.26$  mM MgATP. When the substrate is phosphorylase  $K_{ia} = 0.75$  mM phosphorylase b and  $K_{ib} = 7.8$  mM MgATP.

substrates used. This implies that the mechanism has more steady state character than rapid equilibrium; in other words, the interconversion of the ternary complexes (see scheme, page 71) is not slow compared to the other kinetic steps. Therefore the equation  $K_{ia}K_b = K_{ib}K_a$  which is normally used to calculate  $K_{ia}, K_{ib}$  values cannot be used here and are therefore not included in Table 1. Complex rate equations can be generated with the aid of a computer, to fit the data obtained (Fromm, 1975); however, this is beyond the scope of this work. For the present work it is considered sufficient to know the mechanism and kinetic parameters  $K_a, K_b$  and  $V_1$  for the phosphorylase kinase reaction.

From the kinetic mechanism of phosphorylase kinase it is concluded that there exists an enzyme-MgATP-peptide complex. The Michaelis constants of MgATP and peptide and the maximal velocity ( $V_1$ ) under saturating conditions of both substrates are now known. The questions that can now be asked are the following: what constitutes a good minimal length peptide? What substitution or deletion can be tolerated? Are these expressed in terms of the affinity of peptide for enzyme ( $K_m$ ) or only in the catalytic efficiency ( $V_m$ ), or both? On the basis of the kinetic experiments it is now possible to provide at least a partial explanation.

Various peptides were used in an attempt to answer these questions. From studies involving substrate specificity of

Ser-asp-gln-glu-lys-arg-lys-gln-ile-ser-val-arg-gly-leu

1	2	3	4	5	6	7	8	9	10	11	12	13	14
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small peptides, the following characteristic features were observed. The hexapeptide (sequence 9-14) was observed to be a substrate for activated phosphorylase kinase, although a poor one, with a low  $V_m$  (2% compared to tetradecapeptide). This type of peptide could serve as an interesting model for studying the effect of substitution on the  $K_m$  and  $V_m$  of the reaction. For example, the  $K_m$  of this peptide was as good as that of the reference tetradecapeptide, but what effects would be seen on the kinetic parameters if isoleucine, or valine, or arginine were substituted by glycine? Evidence has been presented with preincubated nonactivated kinase and larger peptides (5-14) that the effect of such substitutions are important in that the  $V_m$  is lowered (Tessmer, 1975). Other interesting possibilities for substitutions are the isomer and analogue, allo-isoleucine and canavanine of isoleucine and arginine, respectively. The former contains a change in the orientation of the side chain; the latter a change in the pK of the guanidinium group.

When an arginine residue is the C-terminal amino-acid in a peptide 1-12 (Tessmer, 1975) the  $K_m$  was decreased by a factor of 6 and the  $V_m$  by a factor of 15 as compared to

Ser-asp-gln-glu-lys-arg-lys-gln-ile-ser-val-arg-gly-leu

1 2 3 4 5 6 7 8 9 10 11 12 13 14

---

tetradecapeptide. Thus a negative charge on arginine in a C-terminal position cannot be tolerated, suggesting that arginine of the substrate plays some critical role in the catalytic reaction. Table 4 shows three hexapeptides with arginine C-terminals. The relative  $V_m$  of the hexapeptides are increased by removing the negative charge of arginine by replacing the hydroxyl group by an amide group. Further increase in  $V_m$  is noted when the positive charge on lysine is replaced by an acetyl group. In this respect it is interesting to note that a hexapeptide (9-14, Table 4) binds as well as tetradecapeptide (1-14) although the relative  $V_m$  is only 2%. Thus two aspects are important, size of the peptide and an arginine residue in position 12.

A conservative substitution of arginine by lysine at position 12 lowers the catalytic efficiency considerably. The same result is observed when alanine is substituted for arginine. Since the latter is a nonconservative substitution this is not completely unexpected (Tessmer, 1975). The poor extent of phosphorylation observed with peptide is primarily due to the change in the rate of catalysis ( $V_m$ ) than to a change in  $K_m$  of the peptide for phosphorylase kinase (Table 5). However, to establish that the results are not due to

subtle changes in the apparent Michaelis constants, a more complete kinetic experiment should be done whereby the Michaelis constants are determined under saturating conditions of both peptide and MgATP, i.e., the determination of the  $K_a$ . The reason for this is the following. In a two substrate system (the phosphorylase kinase reaction) the  $K_m$  of peptide is affected by the MgATP concentration and, vice versa, the  $K_m$  of MgATP depends on the peptide concentration used. Extrapolation of the initial velocities data to infinite substrate concentration results in an intersection point, from which the  $K_a$  can be evaluated.

From the results of the specificity studies we found that arginine (position 12) is important in the catalytic reaction; although it is realized that this residue is certainly not the only important one as discussed earlier. Possible roles for the involvement of arginine could include a specific binding site for the arginine guanidinium group in the kinase catalytic site; and substitution of lysine for arginine causes the peptide to assume an unfavorable conformation. A second possibility could be that substrate arginine forms a hydrogen-bonded complex with the  $\gamma$ -phosphoryl group of ATP bound in the active site of phosphorylase kinase. In fact, existence for a guanidinium group phosphate complex has been used (McLaughlin et al., 1976) to explain the binding of MgATP in the creatine kinase active site. A direct

role for arginine in the catalytic reaction could be through an intermediate step involving  $\gamma$ -phosphoryl group transfer to substrate arginine, followed by a transfer step of  $\gamma$ -phosphoryl to the serine residue. The fact that reversibility of the kinase reaction could not be demonstrated could mean that either one of these steps is kinetically unfavorable for the reversal reaction, but not in the forward reaction.

The observation that the 12-lys peptide is such a poor substrate compared to a peptide containing a 12-arg is interesting from an evolutionary point of view. Trypsin, for example, recognizes either amino acid in a substrate; alkaline phosphatase requires divalent metal ions while phosphorylase phosphatase requires an arginine residue at position 12 of tetradecapaptide (Martensen et al., 1973). Martensen et al., (1973) reported that divalent metal ions and a guanidinium analogue behaved as competitive inhibitors of the dephosphorylation of P-tetradecapaptide and phosphorylase a. These enzymes evidently contain a binding site for a positively charged ligand. This can either be satisfied (in the examples above) by divalent metal ions or by the bivalent guanidinium group of arginine.

As mentioned earlier, measurements of circular dichroism of peptides could be used to determine whether or not peptides have "structure", i.e., a particular conformation (helical,  $\beta$ -sheet or random). Unpublished data for

tetradecapaptide have shown that this peptide gives a strong signal which is destroyed by structure destroying substances such as guanidine hydrochloride. The establishment of a relationship between the circular dichroism measurements of the peptides to the kinetic parameters of the kinase reaction with these peptides, might indicate that there could be a similar conformation of the corresponding peptide segment in the intact phosphorylase b molecule. However, it was pointed out earlier that protein kinase cannot phosphorylate phosphorylase b, but can phosphorylate various peptide substrates of phosphorylase kinase (unpublished results). Thus any criteria regarding specificity of phosphorylase kinase relative to peptides and phosphorylase b must necessarily also agree with criteria of specificity of protein kinase relative to peptides and phosphorylase b. If this condition cannot be satisfied it is conceivable that not only the amino acid sequence and conformation of the peptide segment in question is important in determining specificity, but also the interactions of the folded polypeptide chain of the macromolecule with the peptide segment that serves as substrate for phosphorylase kinase.

#### Future Work

This work has but only scratched the surface concerning the problem of how an enzyme such as phosphorylase kinase



recognizes its protein substrate phosphorylase. The kinetic analysis of the kinase reaction as well as the specificity studies done with peptides, at least have given us some idea about some of the aspects of phosphorylase kinase specificity. However, the real test will be on how to relate this in terms of the specificity seen with phosphorylase b as substrate, especially with reference to protein kinase specificity. Some ideas on how this problem may be approached are discussed below.

1. Modification of a "good" peptide by tagging on to the end an extended hydrophobic alkyl chain might anchor the peptide to the kinase molecule, through hydrophobic interactions, and thereby improve the  $K_m$  of peptide. The question is, will the  $K_m$  now approach that of phosphorylase b? It should be pointed out that although the  $V_m$  was only one half of that with phosphorylase b as substrate, the  $K_m$  was 64-fold greater. Another possibility could be that the hydrophobic chain might confer some type of structure to the peptide (helical,  $\beta$ -sheet or random) which would result in a lowered  $K_m$  for the kinase. The matter of structure can be checked with circular dichroism measurements.

2. The neutral amino acids on either side of the phosphorylatable serine residue have also been shown to be important, inasmuch as the peptide's ability to serve as substrate for phosphorylase kinase is adversely affected by

substitution at this position. The question is, will substitution by a neutral amino acid such as the isomer of isoleucine, alloisoleucine, be tolerated? Would this segment of the peptide now have the wrong conformation so that serine can now no longer be phosphorylated? Would protein kinase be able to phosphorylate this peptide?

Substitution of arginine (in position 12) by canavanine should also be tried. Canavanine is interesting because the pK of its guanidinium group is lower than that of arginine (pK 7 and pK 12.5, respectively).

In the final analysis, one would still have to relate results of specificity studies to the known specificities of phosphorylase kinase and protein kinase in regard to phosphorylase b.

3. The  $V_m$  of the phosphorylase kinase reaction using the alanine-substituted peptide was low. As discussed earlier, it is possible that phosphorylase kinase contains a site for the positively charged guanidinium group. Can the  $V_m$  of the reaction with the alanine-substituted peptide be increased by the proper free  $Mg^{2+}$  ion concentration, or by the proper guanidino compound?

4. Limited tryptic attack of phosphorylase produced a species of phosphorylase called phosphorylase b', of which the N-terminal peptide has been removed. It is also known that addition of phosphotetradecapeptide to phosphorylase b'

restores 80% of the AMP independent activity. Furthermore, unpublished results have shown that dephosphotetradecapeptide increases the AMP dependent activity of phosphorylase b'. The question is, if there is sufficient binding of tetradecapeptide to phosphorylase b', (by association only, no covalent bonds) will protein kinase phosphorylate this peptide (taking into account that unbound peptide would be phosphorylated). Is the conformation of peptide similar to that in native phosphorylase? As the X-ray crystallographic work of phosphorylase b becomes available, it should increase our understanding of the conformation of the N-terminal segment and hence our understanding of protein-protein interaction.

5. If it is possible to produce and isolate antibodies to the N-terminal portion of the intact phosphorylase, then these could perhaps be used to "freeze" the conformation of tetradecapeptide (derived from the N-terminal segment) into the correct conformation. Would this stable antibody-peptide complex serve as substrate for phosphorylase kinase; and for protein kinase.

6. Two models have been proposed based on the role of arginine in the catalytic mechanism of phosphorylase kinase; i: substrate arginine binds to a charged group on kinase; ii: substrate arginine aids in stabilizing the transition state of the  $\gamma$ -phosphoryl group of ATP bound in the active site of phosphorylase kinase. Experiments could be done with

specific reagents for arginine, such as cyclohexanedione, to modify arginines of kinase, hopefully one of which will be in the active site. Determination of the kinetic parameters of modified enzyme with tetradecapeptide and phosphorylase b should be compared to the kinetic parameters of unmodified enzyme with modified peptide and phosphorylase b. Information of this type might help in distinguishing between the roles for arginine mentioned above.

7. Another suggestion which has been made is to study structure of "good" and "bad" peptides by nuclear magnetic resonance measurements (NMR). These would be supplementary to measurements made by circular dichroism as mentioned under Discussion.

## SUMMARY

This work describes the use of synthetic peptides as alternative substrates to study the kinetic mechanism and specificity of the phosphorylase kinase reaction. Below follows a summary of the results found.

1. The validity of the model system was tested with a synthetic tetradecapeptide substrate and it was found that the phosphorylase kinase reactions in the presence of the alternative substrate were identical to those with its natural substrate, phosphorylase b, in regard to pH profile, lag in the rate of the catalytic reaction, effect of pre-incubation with peptide substrate on the lag, and the effect of kinase concentration on the lag.

2. The lag in the rate of product formation seen with nonactivated kinase could be eliminated by preincubating nonactivated phosphorylase kinase with peptide substrate in the presence of  $\text{Ca}^{2+}$  prior to initiation of the assay with MgATP. This resulted in a 3 to 3.5-fold increase in specific activity of phosphorylase kinase. The initial velocity was found to be proportional to enzyme concentration over a 200-fold range of enzyme.

3. It was found that phosphorylase kinase activated by cyclic AMP-dependent protein kinase catalyzed reaction showed linear progress curves, so that preincubation with

substrate was not necessary. The initial velocity was found to be proportional to enzyme concentration over the 10-fold range tested.

4. Initial rate studies of activated phosphorylase kinase and tetradecapeptide or phosphorylase b are consistent with a Sequential mechanism for the reaction. The  $K_a$  of synthetic tetradecapeptide was 64-fold larger than the  $K_a$  of phosphorylase b (0.8 mM and 0.012 mM, respectively), while the  $V_1$  was only half as large with peptide compared to phosphorylase b (7.14 and 15.6  $\mu$ mole P/min/mg).

5. From studies with substrate analogues as competitive inhibitors of substrates (threonine-peptide for tetradecapeptide and AMPPCP for ATP) it was found that the data were consistent with random addition of substrates, Random Bi Bi mechanism.

6. A small effect of glycogen on the  $V_m$  of the phosphorylase kinase reaction in the presence of tetradecapeptide was noted. A 30 to 40% increase in  $V_m$  was observed in the presence of 0.4% glycogen.

7. A hexapeptide corresponding to sequence 9-14 of tetradecapeptide:

Ser-asp-gln-glu-lys-arg-lys-gln-ile-ser-val-arg-gly-leu  
 1    2    3    4    5    6    7    8    9    10   11   12   13   14

served as substrate for phosphorylase kinase, although with 2% of the relative  $V_m$  of tetradecapeptide. Increasing the

length of the peptide increased the  $V_m$  to 30% of that of tetradecapeptide without affecting the  $K_m$ , for peptide sequence 7-14.

8. Specificity studies using various substituted synthetic peptides indicated that an arginine residue on the C-terminal side of the convertible serine residue is important for catalysis. Replacing this arginyl residue by alanine or lysine raises the  $K_m$  2 to 3-fold, but lowers the  $V_m$  by a factor of 10 and 40, respectively, relative to the reference tetradecapeptide.

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## APPENDIX A. CALCULATIONS FOR FREE MAGNESIUM IONS

Nucleotide dependent reactions require a 1:1 complex of divalent metal ion and nucleotide as the true substrate (Fromm, 1975). The following calculations were based on the equations and stability constants for Mg-anion complexes presented by Fromm (1975). This reference should be consulted for a complete discussion on factors affecting the stability constants.

1. If a constant level of 10 mM free  $\text{Mg}^{2+}$  is desired then the total  $\text{Mg}^{2+}$  can be calculated at any  $\text{ATP}^{4-}$  concentration from

$$K = \frac{(\text{MgATP}^{2-})}{(\text{Mg}^{2+})_{\text{free}} (\text{ATP}^{4-})_{\text{free}}} \quad (1)$$

where  $K = 20,000 \text{ M}^{-1}$  for  $\text{MgATP}^{2-}$  in 50 mM Tris·HCl (pH 7.6) also,

$$\text{Mg}_0^{2+} = \text{Mg}_{\text{total}}^{2+} = \text{Mg}_{\text{free}}^{2+} + \text{MgATP}^{2-} \quad (2)$$

and

$$\text{ATP}_0^{4-} = \text{ATP}_{\text{total}}^{4-} = \text{ATP}_{\text{free}}^{4-} + \text{MgATP}^{2-}. \quad (3)$$

If the optimal  $\text{Mg}^{2+}$  ion concentration is known then substitution of (2) and (3) in (1) and setting  $\text{MgATP}^{2-}$  equal to  $x$ ,  $\text{MgATP}^{2-}$  can be evaluated from the resulting quadratic equation. Free  $\text{Mg}^{2+}$  can then be obtained by substitution in (2).

2. If  $\text{Mg}_{\text{free}}^{2+}$  is 10 mM, and  $\text{ATP}^4 = 1.5 \text{ mM}$ , then  $\text{MgATP}^{2-}$

can be calculated by the substitution of (3) into (1)

$$K = \frac{(\text{MgATP}^{2-})}{(\text{Mg}^{2+}_{\text{free}}) (\text{ATP}_0^{4-} - \text{MgATP}^{2-})} \quad (4)$$

$$\text{MgATP}^{2-} = 1.49 \text{ mM} .$$

3. Total  $\text{Mg}^{2+}$  needed is calculated from (2)

$$\text{Mg}_0^{2+} = \text{Mg}_{\text{free}}^{2+} + \text{MgATP}^{2-}$$

$$\text{Mg}_0^{2+} = 10 \text{ mM} + 1.49 \text{ mM} = 11.49 \text{ mM}.$$

## APPENDIX B. RATE EQUATIONS

The rate equation for the Sequential mechanism is described by the following equation (Fromm, 1975)

$$\frac{1}{v} = \frac{1}{V_1} + \frac{K_a}{V_1 A} + \frac{K_b}{V_1 B} + \frac{K_{ia} K_b}{V_1 AB} \quad (1)$$

$v$  is the initial velocity;  $V_1$  is the maximal velocity in the forward direction at infinite substrate concentrations of A and B tetradecapeptide and MgATP, respectively;  $K_a$  and  $K_b$  are the Michaelis constants at infinite concentration of both substrates;  $K_{ia}$  is the enzyme-substrate dissociation constant (see scheme, p. 71).

When the reciprocal of the initial velocity is plotted versus the reciprocal of substrate concentration (see Figure 9), the intercept of Equation (1) is described by the following equation

$$\text{Intercept} = \frac{1}{V_1} \left[ 1 + \frac{K_b}{B} \right] \quad (2)$$

and the slope of Equation (1) is described by

$$\text{slope} = \frac{1}{V_1} \left[ K_a + \frac{K_{ia} K_b}{B} \right]. \quad (3)$$

When the reciprocal of the initial velocity is plotted versus the reciprocal of the second substrate (B), as shown in Figure 10, the intercept of Equation (1) is then

described by

$$\text{Intercept} = \frac{1}{V_1} \left[ 1 + \frac{K_a}{A} \right] \quad (4)$$

and the slope is described by

$$\text{Slope} = \frac{1}{V_1} \left[ K_b + \frac{K_{ia} K_b}{A} \right]. \quad (5)$$

The intercept and slope plots are called secondary plots. From the plots described by these equations (see Figures 11 and 12) the  $K_a$  and  $V_1$  can be evaluated. From Equations 2 and 4 (Figure 11),  $V_1$  is the intercept and the slopes are described by  $K_b/V_1$  and  $K_a/V_1$ , respectively.

From the slope equations (3) and (5), and Figure 12, an additional quantity,  $K_{ia}$ , can be calculated. From the slope of the equation described by (3)

$$\text{Slope} = \frac{K_{ia} K_b}{V_1} \quad (6)$$

$K_{ia}$ , the only unknown quantity can be calculated.

Only for a truly Rapid Equilibrium system the following identity is true

$$K_{ia} K_b = K_{ib} K_a \quad (7)$$

and  $K_{ib}$  can be calculated.

The phosphorylase kinase reaction was not found to be



truly Rapid Equilibrium as the  $K_{ia}$  and  $K_{ib}$  values depended on the substrates used (see Discussion).